Studies on Weak Organic Acid Resistance in Yeast

by

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Abstract

Certain yeasts can grow at low pH in the presence of the highest levels of weak organic acid preservatives permitted in foods, leading to spoilage of many manufactured products. This study sought to gain further understanding into the weak acid mechanisms of the yeast *Saccharomyces cerevisiae*, to help identify novel routes to inhibit the growth of this yeast.

Weak acids are thought to act by reducing intracellular pH (pH_i), thus disrupting cellular homeostasis. To definitively link pH_i to weak acid inhibition, a new fluorescent in-vivo pH_i method was developed (Chapter 3) to study the effects of this stress on pH_i and growth. The inhibitory action of sorbic acid was found to cause disruption of pH_i, necessitating induction of an energy-consuming response to rebalance homeostasis. The latter in turn reduced the available free energy for growth and division.

To determine other key factors involved in yeast weak acid resistance, the proteome of cells under sorbate stress was investigated using high-resolution two-dimensional gels (Chapter 4). This identified eight cytosolic proteins that were up-regulated. However, contrary to hypothesis, phenotypic evaluation of the knock out deletion mutants of the identified proteins revealed that none were hypersensitive to sorbate.

To gain further insight into these results, the two genetically almost identical strains used in the above proteomic and phenotypic studies were compared. Results showed that the strain used for the proteomic study (Chapter 4) was far more sorbate sensitive than the other used for the physiological measurements (Chapter 3). Since the only major difference was that the former was a tryptophan auxotroph and the latter a tryptophan phototroph, it was investigated in Chapter 5 whether this increased weak acid sensitivity was due to the auxotrophic requirement for tryptophan (i.e. the strain was *trp*-). High levels of exogenous tryptophan suppressed this enhanced sensitivity of *trp*- cells (Chapter 5). It is apparent therefore that increased weak acid sensitivity arises because the acids strongly inhibit the uptake of the aromatic acids from the medium, rendering *trp*- cells extremely sensitive to their requirement to catalyse this uptake.

This identification of this *trp*- effect has implications for the choice of strains used for future weak acid studies. It has allowed us to reassess the findings of previous studies, where gene functions have been assigned based upon studies using *trp*- strains.

Forward

The majority of this work was carried out in the Microbiology department, Unilever Research Colworth, Colworth House, Sharnbrook, Bedfordshire, MK44 1LQ under the supervision of Prof. Peter Piper (UCL) between October 1996 and January 2004 and of Dr P.J. Coote between October 1996 and March 1999. The work was entirely funded by Unilever Research Colworth.

Four papers (listed below) arising from this work have been accepted for publication and include material from Chapters 2, 3, 4, and 5 of this work.

Three of the papers were published under former name of D. Bracey.

Bauer, B.E., **Rossington, D.**, Mollapour, M., Mamnun, Y., Kuchler, K., and Piper, P. (2003). Weak organic acid stress inhibits aromatic amino acids uptake by yeast, causing a strong influence of auxotrophies for these amino acids on the phenotypes of membrane transporter mutants. Eur. J. Biochem. **270**, 1–7.

Bracey, D., Holyoak, C.D., Nebe-von Caron, G. and Coote, P.J. (1997). Determination of the intracellular pH (pHi) of growing cells of *Saccharomyces cerevisiae*: the effect of reduced-expression of the membrane H+-ATPase. J. Microbiol. Methods **31,** 113-125.

Bracey, D., Holyoak, C.D. and Coote, P.J. (1998). Comparison of the inhibitory effect of sorbic acid and amphotericin B on *Saccharomyces cerevisiae*: is growth inhibition dependent on reduced intracellular pH? J. Appl. Micro. **85,** 1056-1066.

Holyoak, C.D., **Bracey, D.**, Piper, P.W., Kuchler, K. and Coote, P.J. (1999). The *Saccharomyces cerevisiae* weak-acid-inducible ABC transporter, Pdr12 transports fluorescein and preservative anions from the cytosol by an energy-dependent mechanism. J. Bacteriol. **181,** 4644-4652.

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Chapter 1 – Introduction

1.1. The history of preservative use in the food industry

Food preservation methods have been used for thousands of years. The earliest techniques included drying, curing, salting, fermenting and cooking, enabling the food to be stored for longer during times of food shortage. More recent preservation methods are still based upon many of the ancient procedures mentioned above, which were used without any knowledge of how they protected foods from spoilage (Anon, 1986, and Gould, 1989). Such antimicrobials are present in herbs and spices, such as pepper, onion and garlic; also as the salts and sugars in foods, such as in fruits and cured meats (Piper *et al.,* 2001, and Thakur *et al.,* 1994). Other documented examples include the use of sulphite for the sterilisation of wine vessels and acids from wine fermentation by-products in pickling (Stratford & Anslow, 1996), the latter are weak acids. The inorganic acid sulphite and the organic acids, benzoate, sorbate, propionate and acetate constitute the preservatives most widely used in the food and beverage industry. They tend to be used when spoilage from yeast and moulds is a problem in low pH products such as, low-fat spreads, cheese, dressings and tea-based beverages. However, as well as foods, products marketed within the Home and Personal Care Business, such products as shampoos, creams and lotions, facial products such as toners, also require the addition of weak organic acids to prevent spoilage. Even with the addition of such chemicals, it is estimated that the UK food industry suffers losses in excess of £100 million each year due to microbial spoilage (Booth & Kroll, 1989).

Today, consumers are increasingly demanding higher quality convenience foods with longer shelf lives, necessitating the need for an increased use of preservatives (Anon, 1986). But the market is also demanding more natural, less additive based products that are nutritionally healthier, i.e. contain less salt, sugar, saturated fats, more polyunsaturated fats and have a lower calorie content, etc (Brul & Coote, 1999). These trends have major implications for food preservation, because these demands mean using less heat, less drying, less salt, less sugar, less acid, less use of additives and unfortunately making effective preservation more difficult (Gould, 1989). Therefore at present, in order to provide satisfactory shelf life, higher preservative

levels have to be added to some of these "natural" foods. But even with their presence, many yeast and moulds can still survive, adapt and grow, even at preservative concentrations at the maximum permitted level, thus posing major problems (Steels, 1999).

Safety issues relating to the increased use of preservatives in foods have recently been given much publicity. An increased understanding of the effects of many preservatives has led to changes to the legislation for their use. For example, studies reporting the risk of possible adverse health effects to the use of sulphites in foods especially in steroid dependant asthmatics, have led to the US Food and Drug Administration (FDA) banning the use of sulphites in some foods (Thakur *et al.,* 1994). The results of other evaluations have meant that many countries have decided only to allow sorbic acid as a permitted organic acid food preservative. The maximum permitted level of sorbate allowed in food products varies greatly from between 1000 and 2000 parts per million (PPM) or 9 to 18 mM (Earle & Putt, 1984). Due to traditional preservatives being subject to increasing restrictions, the search is now on for alternatives. However, for any new antimicrobial to be approved it must pass far stricter standards as set out by the new European Biocides Directive. It is becoming all the more important to address this whole area of preservative use and overcome the spoilage problems caused by preservative-resistant organisms. Thus the principal objective of this thesis is to contribute to a growing base of fundamental knowledge on aspects of yeast physiology and the mechanisms of preservative resistance.

1.2. The role of weak acids as preservatives

Organic weak acids and their esters are naturally found in many foods, especially in fruits or as microbial by-products from fermentation. The organic acids that tend to be active as preservatives have pKa's between 3 and 5 and their activities have been shown to be very pH-dependent. Lowering the pH increases the antimicrobial effectiveness of the acid by increasing the proportion that is in an undissociated form (Booth & Kroll, 1989). At higher pH ranges, particularly at pH 5.5 – 6 (the optimal range for food poisoning bacteria), most organic acids are relatively ineffective as

inhibitors of microbial growth. However, the exceptions to this are sorbic acid and parabens, where sorbate continues to exert an effect up to pH 6.5 (Anon, 1986).

1.2.1. The role and action of sorbic acid

Sorbic acid (trans-trans, 2,4-hexadienoic monocarboxylic acid: CH_3-CH=CH-CH=CH-COOH) occurs naturally as parasorbic acid in the rowan berries of the mountain ash tree. It was discovered in 1859 by a German chemist and later in 1900, chemically synthesised. However, it was not until 1939 that its antimicrobial properties were discovered. Since then it has been permitted for use as an effective preservative (Sofas & Busta, 1981 and Thakur *et al.*, 1994) and is the only unsaturated organic acid permitted in foods (Anon, 1986). A survey conducted by Sofas & Busta (1981) shows how extensively used this preservative is across many different food products, from dairy to bakery, from fruit and vegetables to other preserved products such as mayonnaise. Sorbic acid reacts readily to form salts and esters. These salts, especially potassium salts, are important due to their high solubility in water (Sofas & Busta, 1981).

Considerable research has been conducted to investigate the safety of this compound, much of the early work concluding that the material was non-toxic. However, by the 1950's it was recognised that sorbic acid could be metabolised by humans and some mould species, although *S. cerevisiae* and lactic acid bacteria cannot utilise this compound (Melnick, 1954, Earle & Putt, 1984 and Sofas & Busta, 1981); the spoilage yeast *Zygosaccharomyces bailii* can break it down in the presence of oxygen (Mollapour & Piper, 2001a). The antimicrobial action of sorbate (pKa 4.76) increases with decreasing culture pH showing that the undissociated acid is the active form (Booth & Kroll, 1989). Because sorbic acid has a pKa of 4.76, it dissociates almost completely upon entry into the cell, resulting in the release of protons and anions. As a consequence of this, some studies have proposed that both intracellular pH (pH_i) reduction and accumulation of toxic levels of anions are responsible for the growth inhibition (Salmond *et al.*, 1984 and Cole & Keenan, 1987). However, a low pKa is not the only explanation because both acetate and sorbate, have similar pKa values, but have very different inhibitory activities (Salmond *et al.*, 1984).

1.2.2. The role and action of acetic acid

Wine, beer or any liquid containing less than 18% alcohol becomes vinegar when airborne bacteria such as *Acetobacter aceti* are present to convert the alcohol into acetic acid. The discovery of vinegar as a natural by-product of alcoholic beverage production was almost certainly accidental. Ancient civilisations as far back as the Sumerians used vinegar as a condiment, a preservative, a medicine, as well as a detergent (Anon, 1986). Acetic acid (ethanoic acid; CH_3COOH) is present at 5-10% in vinegar or 25-80% as synthetic acetic acid (Eklund, 1989).

In the 16th century, a German alchemist described the dry distillation of lead and cupric acetates to produce glacial acetic acid and by this the means to vinegar. The presence of water in vinegar had such a profound effect on acetic acid's properties that for centuries glacial acetic acid and the acid found in vinegar were believed to be two different substances. It was not until the 1800's that a French chemist proved them to be identical, and in 1847, the German chemist Hermann Kolbe synthesised acetic acid from inorganic materials for the first time. In the mid 19th century Louis Pasteur published the most modern scientific research on vinegar, bringing about the process for commercial production of vinegar. Most acetic acid made for industrial use is made by one of three chemical processes: butane oxidation, acetaldehyde oxidation, or methanol carbonylation (Nationmaster Encyclopaedia Web-site).

As with sorbic acid, much of the study into the safety of acetic acid use was only conducted very recently. Vinegar, acetic acid and the acetic acid salts (sodium acetate, calcium acetate and calcium diacetate) are quoted as generally regarded as safe to eat (i.e. GRAS listed) and thus are routinely used as food preservatives in many culinary products and sauces. According to the International Commission for Microbiological Specifications for Foods (ICMSF, 1980), 0.1% acetic acid inhibits most food poisoning and spore forming bacteria, whilst 0.3% of the undissociated acid form is needed to control the growth of mycotoxigenic moulds (Anon, 1986). Thus, the primary target of acetic acid tend to be bacteria and yeasts, but the acid is considered to only have limited action linked to its ability to reduce the pH of the external medium (Eklund, 1989).

1.3. The effect of pH on growth and viability

The survival and growth of microbes in both natural and preserved foods is complicated, and affected by a wide range of factors, including; the range and availability of nutrients, temperature, water activity, oxygen partial pressure as well as pH. Spoilage can be delayed or prevented by adjusting one or more of these to a sub-optimal level, so that growth of the normal spoilage micro-flora is inhibited. Many traditional preservation methods rely upon using one of these parameters to extend the shelf life of such products, for instance the use of pH alone to control microbial spoilage of food products. However, using low pH and combinations of heat and/or organic acids can give rise to synergies which can give a greater than-expected stability of foods. Products that contain a higher amount of acid and having a pH lower than 4.5, are far more stable and are usually only at risk of spoilage from yeast and moulds (Booth & Kroll, 1989). It is believed that these preservatives elicit their effect by either reducing or eliminating microbial activity, by disrupting the membrane proton gradients by permeating the cell structure, causing an extended lag phase thus inhibiting rather than killing the cells (Stratford & Anslow, 1996, and Eklund, 1989).

Many types of produce (especially fruit) naturally contain organic acids such as acetic, benzoic, citric, malic, sorbic, succinic, and tartaric acids, in amounts significant to negatively affect the viability of contaminating microbes. Fruits such as melons and papayas contain lower concentrations of organic acids and, being therefore at pH values above 5.0, are more at risk from attack from the spoilage bacteria (Food and Drug Administration, USA, 2001).

1.3.1. Regulation of intracellular pH

It is increasingly recognised that the cell exerts tight regulation over its intracellular pH (pH_i), and that this maintenance of pH_i is essential for growth. From pH homeostasis studies, optimum pH_i is species dependent, but it is also clear that organisms have different capabilities to regulate their cytoplasmic pH, and possibly have different tolerances in the perturbation of pH_i. Yeast cells are known to maintain their pH_i within a narrow physiological range (between approximately 5.0

and 6.5), even though they can still grow when external pH is as low as 2-3 (Cimprich *et al.,* 1995, and Imai & Ohno, 1995).

In order for yeast cells to maintain this constant pH_i, protons are actively extruded from the cytoplasm by the membrane bound H^+-ATPase, an enzyme that generates a trans-membrane proton gradient (the proton-motive force). This pump is energy requiring, so that when a cell is present in a more acidic environment, this pump can consume large amounts of ATP just to maintain pH_i homeostasis. It has been clearly established that increased H^+-ATPase activity is essential for the maintenance of pH_i in low external pH environments and for counteracting sorbate stress of *S. cerevisiae* (Holyoak *et al.*, 1996). However, some microbes which have adapted to growth at low external pH's, have a relatively low pH_i, thus enabling them to exist without the need for such high energy outputs (Booth & Kroll, 1989).

Minor fluctuations in pH_i are believed to play a regulatory role by mediating many important cellular processes (reviewed in Busa & Nuccitelli, 1984). For example, changes in pH_i have been suggested to play a regulatory role in the cell cycle, as a higher pH_i increases rates of DNA and RNA synthesis (Madshus, 1988) and a reduction in pH_i has been suggested as a potential trigger for the heat shock response (Coote *et al.*, 1991).

1.3.2. The effect of intracellular pH on cellular metabolic regulation

Many of the enzymes involved in glycolysis and energy production for the cell are affected by pH_i. Phosphofructokinase (PFK) has been shown to be very sensitive to pH, and it has been suggested that inhibition of this enzyme is responsible for the growth inhibition caused by different weak acids (Francois *et al.,* 1986). Indeed, it is believed that one mode of action of weak-acid preservatives is to reduce pH_i below the normal physiological range, leading to growth arrest by inhibition of glycolytic flux at the PFK reaction (Krebs *et al.,* 1983). This inhibition leads to trehalose accumulation in sorbate stressed *S. cerevisiae* (Cheng *et al.,* 1999).

1.4. The inhibitory mode of action of weak acid preservatives

Weak organic acids exist in solution in a dynamic equilibrium between undissociated, uncharged molecules and their respective dissociated charged anions ($XCOO^-$) and protons (H^+). Such an equilibrium is pH dependent; and thus at low pH the weak acid will exist substantially in the undissociated state (XCOOH), a form which is a potent inhibitor. For example, sorbic acid at pH 3, 98% exists in the undissociated form, whereas at pH 7, just 0.6% is in a similar state (Earle & Putt, 1984). All weak organic acids inhibit more strongly at low pH, inhibiting rather than killing microbes. Where growth is possible, it occurs after a prolonged lag phase, biomass yields are reduced and active transport is prevented (Stratford & Anslow, 1998). At neutral pH, when the weak acids are almost completely dissociated, very high concentrations can still be inhibitory to *S. cerevisiae* (Piper *et al.,* 2001).

Figure 1.1 depicts a model for the mechanism of yeast inhibition by carboxylic acids. The undissociated acid, being uncharged, freely diffuses across the cell membrane only to dissociate once in the higher near-neutral pH environment of the cytosol. Thus to be effective the weak acid must be able to pass through the two major barriers protecting the yeast cell from adverse environmental change, the cell wall and the plasma membrane. Once inside the weak acid dissociates generating protons and acid anions acidifying the cytoplasm. The cell responds by increased plasma membrane protein H^+-ATPase (Pma1p)-catalysed proton extrusion. The acid anion will tend to accumulate intracellularly to high levels as it cannot readily diffuse out of the cell. This high anion accumulation can potentially generate high turgor pressure within in the cell and, in the case of aerobic, sorbate-treated yeast, enhance free radical formation leading to oxidative stress (Piper *et al.,* 2001). A combination of intracellular pH reduction by the dissociation of hydrogen ions from the acid, disruption of membrane transport and/or permeability, anion accumulation will lead to cell inhibition (Piper *et al.,* 2001).

1.4.1. Weak acid targets within the cell

Piper *et al.* (2001) discussed a number of studies that have compared different weak acid effects. The mode of action of weak acid preservatives cannot be solely due to this effect of pH_i leading to growth inhibition. Instead the inhibitory action via the membrane increases, as the weak acids become more lipophilic. Preservatives with identical pKa (e.g. acetic and sorbic acid; both have a pKa of 4.76) have very different antimicrobial activities (Salmond *et al.*, 1984 and Booth & Kroll, 1989). Thus acetate could possibly be solely working to disrupt the intracellular pH (as in Figure 1.1) and decreases in pH_i have been shown in cells on the addition of acetate. In contrast, the more hydrophobic sorbic acid is mainly inhibiting cells through the disordering of the membrane structure. Sorbate addition does not result in a drop in pH_i (Bracey *et al.*, 1998) and in aerobic yeast, generates (unlike acetate) very severe oxidative stress by enhancing free radical formation by the mitochondrial respiratory chain (Piper *et al.*, 2001).

Evidence of a membrane effect in bacteria is reported by Freese *et al.* (1973), where the inhibitory action of sorbic acid may be partly due to an 'uncoupling' effect at the plasma membrane, and as further discussed by Stratford & Anslow (1996). Different types of stress all have a similar effect of increasing passive proton influx, which dissipates the electrochemical potential gradient maintained at this membrane by the H^+-ATPase. Disruption of this gradient adversely affects many vital functions for which this electrochemical gradient is essential, such as amino acid and ammonium ion uptake, maintenance of a potassium balance, but also the regulation of pH_i (Piper, 1995). Growth inhibition can in principle be caused by the interference with the cell wall, membranes, metabolic enzymes, protein synthesis and the genetic material (Eklund, 1989). However, of all the weak acids discussed, only sorbic acid has been reported to disturb the cell wall, which is in agreement with the above discussions.

The literature also indicates specific enzymes might be the targets of inhibition within cells by weak acids. York *et al.* (1964) reported that they had shown the sulfhydryl enzymes; fumarase, aspartase and succinic dehydrogenase were inhibited by sorbic acid in both bacteria and yeast. Other enzymic reactions have also been reported to be sorbic acid targets such as, ficin and alcohol dehydrogenase. As

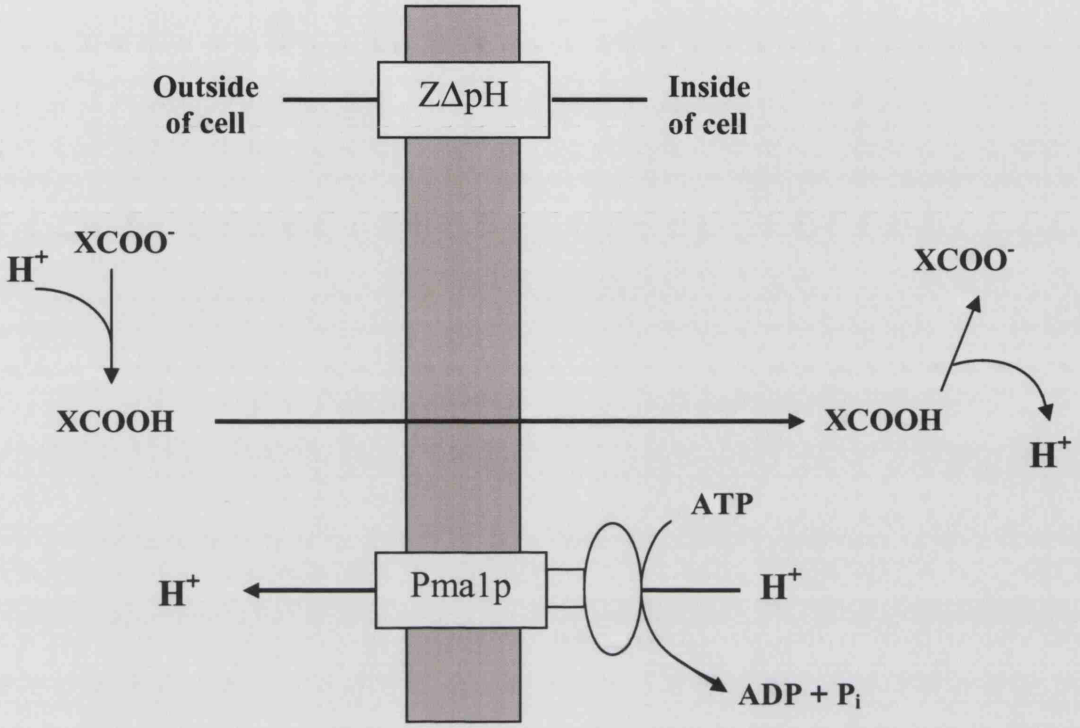
already discussed in section 1.3.2, the glycolytic enzymes and in particular PFK are inhibited by weak acids, thus causing the energy depletions via disruption of glycolysis seen when sorbate or benzoate are added to cells (Krebs *et al.*, 1983).

1.5. Weak acids can induce different yeast stress responses

Many different studies have investigated the resistance responses of yeast to weak organic acids. Sorbate stress leads to strong induction of a number of genes, by the activation of Msn2/Msn4p (de Nobel *et al.,* 2001) and War1p transcription factors (Kren *et al.*, 2003). However the Msn2/Msn4p activation is not important for acquisition of sorbate resistance (Hatzixanthis *et al.*, 2003) and it appears *PDR12* is the only War1p-regulated factor needed for the pronounced effects of War1p loss on sorbate resistance (Schüller *et al.*, 2004). This is despite several other activities in addition to Pdr12p, activities that are not War1p-regulated, being required for resistance.

1.5.1. The role of H^+-ATPase (Pma1p) in the response to weak acid stress

Besides these changes in gene expression, physiological changes are also important. Yeast use a dedicated enzyme for the maintenance of pH_i homeostasis via the activity of a proton translocating plasma membrane H^+-ATPase which couples ATP hydrolysis to the expulsion of protons thus generating the proton motive force (Serrano, 1984). The plasma membrane H^+-ATPase is essential for growth and cell viability, a null mutation in haploid cells is lethal (Serrano *et al.,* 1986). In *S. cerevisiae*, this protein is the most abundant plasma membrane protein, constituting over 20% of the total membrane proteins (Holyoak *et al.,* 1996). The increase in its activity with weak acid stress (Holyoak *et al.,* 1996, Piper *et al.*, 1997 and Viegas & Sa Correia, 1991) is important for counteracting the stress, since mutants with a reduced plasma membrane H^+-ATPase activity are sorbate sensitive (Holyoak *et al.,* 1996). An important charge-balancing mechanism for this stress stimulation is that the plasma membrane H^+-ATPase catalysed efflux of protons from the cell, may be K^+-influx, since sorbate sensitivity results from the loss of the Trk1p high affinity K^+ uptake transporter (Bertl *et al.*, 2003) in *trk1*Δ cells (Mollapour, unpublished).



Derived from Piper *et al.* (2001).

Figure 1.1

Schematic representation of the mechanism of the undissociated weak organic acid (XCOOH) in unadapted yeast cells. The protonated uncharged species is freely permeable across the cell membrane, once in the cell it dissociates into the anion ($XCOO^-$) and proton (H^+). In unadapted cells the concentration of the XCOOH gradually reaches equilibrium. In *S. cerevisiae* the electrochemical potential difference across the plasma membrane (ZΔpH) is largely maintained by active extrusion of the protons by the plasma membrane H^+-ATPase (Pma1p). The anion is impermeable to the cell membrane and thus will accumulate inside the cell.

1.5.1.1. H^+-ATPase structure

Plasma membrane H^+-ATPase belongs to the P-type family of cation pumping ATPases that are characterised by the formation of a phosphorylated intermediate. It consists of a single polypeptide chain comprising of 918 amino acids folded into eight trans-membrane α helices with N and C-terminal domains both located in the cytoplasm. Studies show that these helices are amphipathic and thus form polar channels in the membrane through which the cations are pumped (Serrano *et al.*, 1986 and Serrano, 1988). The C-terminal domain is thought to be a potential site of phosphorylation by the Ca^{2+}calmodulin-dependent protein kinase and thus regulation of the enzyme activity. Stoichiometric studies have shown that the protein consumes 1 ATP for every H^+ ion pumped out of the cell (Serrano *et al.*, 1986).

1.5.1.2. H^+-ATPase function

Studies have shown that the activity of H^+-ATPase within *S. cerevisiae* can be rate limiting for growth (Serrano *et al.*, 1986 and Portillo & Serrano, 1989) and activated by glucose. Maintenance of pH_i homeostasis can be energetically expensive, resulting in the protein consuming between 40 to 60% of total cellular ATP present (Serrano, 1988 and 1991). It is conceivable that weak acids not only inhibit glycolysis but also deplete existing cellular ATP levels by forcing the cell to consume ATP in an attempt to maintain pH_i homeostasis (Cole & Keenan, 1987). Weak acid stress will dramatically increase the requirement of ATP for pH_i homeostasis (Figure 1.1), thus explaining why total cell biomass in acid stressed cells increases only slowly, since the energy yields of a high rate of glycolytic flux are mostly used in counteracting the stress (Holyoak *et al.*, 1996). Thus yeast cells can invest a large proportion of cellular energy in maintenance of pH_i homeostasis, confirming that pH_i is of fundamental importance to cell physiology and requires tight regulation (Booth & Kroll, 1989). Studies have shown that when cells are grown within an acidic environment, H^+-ATPase activity is increased (Eraso & Gancedo, 1987).

The plasma membrane H^+-ATPase has also been shown to be important determinant in several stress responses, including the responses to heat and to weak organic acids (Coote *et al.,* 1994 and Panaretou & Piper, 1990). Holyoak *et al.* (1996) suggested that increased weak acid resistance may due in part from a dramatically increased H^+-ATPase activity.

1.5.1.3. H^+-ATPase in ion transportation

The proton motive force generated by H^+-ATPase is important not only for pH_i homeostasis, but also for the active transportation of nutrients. This proton gradient is the driving force for nutrient uptake such that its disruption will adversely affect nutrient intake by the cell, resulting in growth arrest (Portillo & Serrano, 1989 and Eddy, 1982).

1.5.2. Heat shock proteins

The heat shock response is a universal response seen in all organisms when exposed to elevated temperature. In yeast its induction is due to the activation of heat-shock transcription factor (Chatterjee *et al.,* 2000) and leads to the synthesis of a set of evolutionarily-conserved heat shock proteins (Hsps). The latter, in turn, increase the cell's ability to counteract heat stress (Piper, 1995 and Panaretou & Piper, 1992) and, in many instances, other types of stress such as ethanol exposure or starvation. Hsps are divided into two major size classes of 70-100kDa and 17-30kDa and in many organisms these are encoded by multi-gene families. Several different protective functions have been associated with these proteins, including both protein chaperoning and antioxidant defences.

Many heat shock proteins are molecular chaperones, directly involved in protein biogenesis, the assembly of protein structures, and the transfer of proteins across membranes. They block non-productive protein-protein interactions, thereby allowing unfolded proteins to form the correct tertiary structure, and assisting in chaperoning, reactivation and degradation of unfolded proteins (Craig *et al.*, 1993, Panaretou & Piper, 1992 and Piper *et al.,* 1997). The accumulation of denatured

proteins is commonly regarded as the trigger for the Hsp stress response, although a number of studies have shown that the plasma membrane is critical in the transduction of heat stress into a biological signal (Coote *et al.*, 1994).

1.5.2.1. Hsp30, the single integral plasma membrane heat shock protein

Saccharomyces cerevisiae has a single integral plasma membrane heat shock protein called Hsp30. It is a highly hydrophobic membrane protein that has been found to be primarily associated in plasma membrane fractions (Panaretou & Piper, 1992). This protein is induced by many of the stresses listed above for the other Hsps, including heat shock, ethanol exposure, severe osmo-stress, weak acid exposure and glucose limitation (Piper *et al.,* 1994). Studies by Piper *et al.* (1997) and Braley & Piper (1997) revealed that Hsp30 induction leads to down-regulation of the stress stimulation of H^+-ATPase. The plasma membrane H^+-ATPase consumes a substantial amount of ATP generated by the cell in order to maintain cellular homeostasis during times of stress. Thus it was hypothesised Hsp30 may therefore provide an energy conservation role, limiting excessive consumption of ATP by the H^+-ATPase during times of prolonged stress or glucose limitation.

1.5.3. The Pleiotropic Drug Resistance (PDR) multi-drug pump network

Multi-drug resistance in *S. cerevisiae* is a generalised resistance to a broad spectrum of functionally unrelated compounds (Balzi & Goffeau, 1995). Recent analysis has confirmed that a complex network of genes and membrane transporters give tolerance to cytotoxic compounds (Balzi & Goffeau, 1995 and Egner *et al.,* 2000). These proteins have been classified into three major classes: ATP-driven membrane transport proteins, membrane transporters of the major facilitator superfamily (MFS) that use a membrane gradient or potential, and transcription factors (Balzi & Goffeau, 1995). The group of most relevance to this study are the membrane transporters of the ATP-binding cassette (ABC) transporter superfamily, which couple the hydrolysis of ATP to the transport of compounds across the membrane. Yeast has 31 genes for ABC-transporters (Egner *et al.*, 2000), examples being *SNQ2* (required for resistance to nitroxiquinoline, Servos *et al.*, 1993), *PDR5* (resistance to

various anti cancer drugs; Kolaczkowski *et al.*, 1996) and *PDR12* (resistance to weak organic acids, Piper *et al.,* 1998). Most ABC-transporters contain two highly conserved ATP-binding domains, and 12 trans-membrane domains. Their structural organisation has been highly conserved throughout evolution, yet each one appears to transport a specific set of substrates.

1.5.3.1. The role of Pdr12

Exposure of *S. cerevisiae* to sorbic acid strongly induces two plasma membrane proteins, Hsp30 and ATP-binding cassette (ABC) transporter protein Pdr12 (Piper *et al.,* 1998). The sorbate induction of Pdr12p is so strong, that this becomes one of the most abundant plasma membrane proteins (Piper *et al.,* 2001). Cells lacking the Pdr12 transporter (the *pdr12 S. cerevisiae* mutant) are hypersensitive to water-soluble monocarboxylic acids of relatively short aliphatic carbon chain length (Piper *et al.,* 1998, and Holyoak *et al.,* 1999). They are also sensitive to short chain alkanols (n-propanol, n-butanol and n-pentanol) (Hatzixanthis *et al.*, 2003). However *pdr12* mutant cells are not sensitive to dicarboxylic acids; or those highly lipophilic, long chain fatty acids and alcohols whose toxic effects are thought to be mainly due to a detergent disruption effect on membranes (Holyoak *et al.,* 1999, and Weber & de Bont, 1996). It appears therefore that Pdr12 transporter imparts resistance to those organic acids or alcohols that can, to a reasonable degree, partition into both lipid and aqueous phases (Holyoak *et al.,* 1999, and Holyoak *et al.,* 2000). Its loss does not render cells hypersensitive to the known substrates for other well-characterised *S. cerevisiae* ABC transporters, drug pumps such as Pdr5 or Snq2 (Egner *et al.,* 2000). Pdr12 was the first ABC transporter identified as a weak acid pump. Whilst it was originally thought to confer some resistance to acetate (Piper, 1998), it has recently been shown that this is due to the use of strains of *trp1* genetic background and that Pdr12 confers resistance to propionate, but not acetate (Bauer *et al.*, 2003).

1.6. A model of weak acid resistance and adaptation

1.6.1. Examples of weak acid resistance in nature

Many spoilage micro-organisms (mainly bacteria) generally cannot grow at pH values much below pH 4.5 so that acidification can act to prevent microbial spoilage. Products such as fruits, soft drinks, vinegars and wines have generally have much lower pH values, but can still suffer from spoilage from yeasts and moulds rather than from bacteria (Booth & Kroll, 1989). There are some yeast and mould strains that are highly capable of growing at these pH's and lower, and certain strains of *Z. bailii* and *Z. rouxii* can tolerate extreme environments such as low water activity. They are also able to undergo adaptation that allows them to grow in the presence of preservative concentrations in excess of those permitted legally in foodstuffs (Steels *et al.,* 1999). In any *Z. bailii* culture the individual cells differ very considerably in their sorbate resistance, a small fraction being remarkably resistant cells. Nevertheless *S. cerevisiae* isolated from instances of food spoilage can frequently adapt to levels of sorbate and benzoate only slightly lower than the levels inhibitory to *Z. bailii* and *Z. lentus* (Piper *et al.,* 2001).

A number of studies have investigated these extraordinary resistance mechanisms. Some of the strains are capable of utilising the preservative and breaking it down. *Z. bailii,* unlike *S. cerevisiae,* possesses a mitochondrial monooxygenase that enables it to catalyse oxidative degradation of both sorbate and benzoate (Mollapour & Piper, 2001a and Mollapour & Piper, 2001b). *Z. bailii,* also unlike *S. cerevisiae,* can also utilise acetate in the presence of sugars, which could explain some of its ability to tolerate high acetate (Sousa *et al.*, 1996).

1.6.2. Defining weak acid adaptation

Research to demonstrate weak acid adaptation has been shown by Holyoak *et al.* (1996) and also Piper *et al.* (1998). Following the addition of 0.5 - 4 mM sorbate, *S. cerevisiae* cultures in growth at pH 4.5 exit the cell cycle and enter a long period of stasis. Eventually, after a period of several hours to days, they resume growth. In this state their total cell biomass increases only slowly, since the energy yields of a high rate

of glycolytic flux are mostly used in counteracting the stress. The cells are though now stress-adapted, in that they will not display another transient growth arrest if re-inoculated into fresh pH 4.5 medium containing the same, sub-inhibitory levels of sorbate (Holyoak *et al.*, 1999, Piper *et al.,* 1998 and Piper *et al.,* 1997).

1.6.3. A model for the adaptation of yeast to weak acids

Adaptation to a stress involves both immediate and longer-term components. Holyoak *et al.* (1996) showed that H^+-ATPase plays a critical and energy-demanding role in adaptation to weak acid stress. This is supported by the findings of Cole & Keenan (1987) that also implicate the H^+-ATPase activity as one possible mechanism involved in weak acid resistance. As already discussed in section 1.5.1.2., acidification of the cytosol due to the intracellular dissociation of the weak acid could be counteracted by this increased activity of the H^+-ATPase which catalyses proton extrusion from the cell, as depicted in Figure 1.1 (Piper *et al.,* 2001).

Studies into such increase in activity with relevance to different stresses include heat (Coote *et al.*, 1991), ethanol exposure (Cartwright, *et al.,* 1986) and weak acid treatment (Holyoak *et al.,* 1996, and Viegas & Sa-Correia, 1991). It is known that the H^+-ATPase consumes much of the ATP generated by the cell, as such maximal activity is rare and energetically expensive to maintain (Serrano, 1991). Thus high activity of this membrane protein over long periods of time might be unsustainable (Piper *et al.,* 1997).

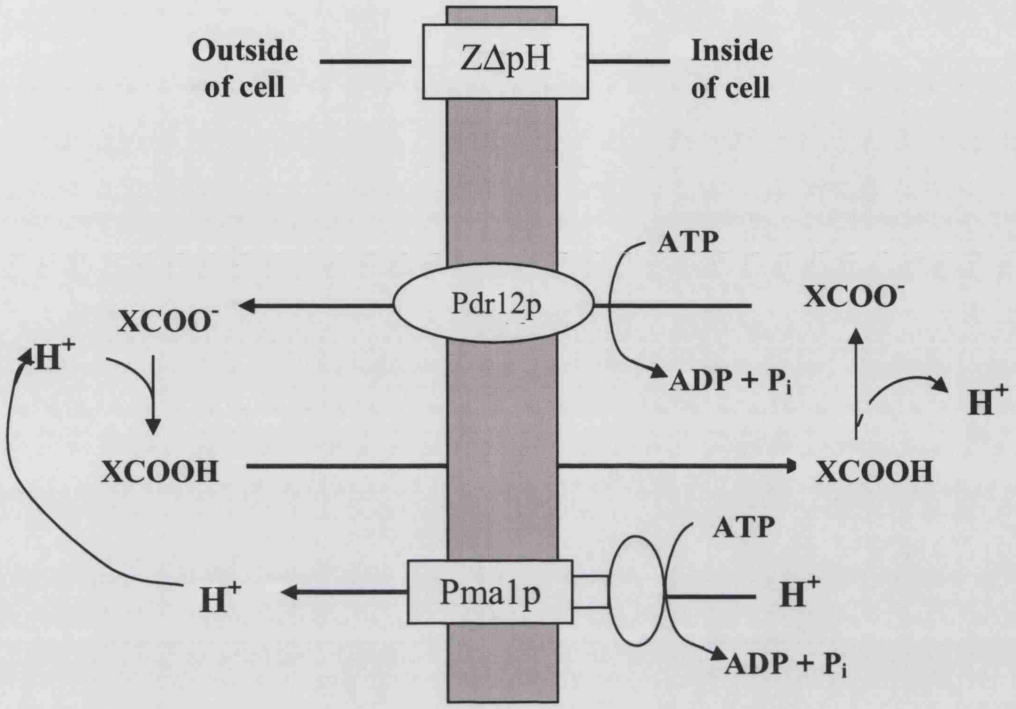
An immediate response to weak acid stress seems to be the increase in the H^+-ATPase activity, but this is followed up by a delayed response in the induction of Hsp30. Piper *et al.* (1997) demonstrated that Hsp30 partially down regulates the sorbate induced increase in H^+-ATPase activity, presumably limiting excessive energy consumption and thus conserving ATP during extended weak acid exposure. It is conceivable that Hsp30 might partly occlude the C-terminal regulatory domain of the H^+-ATPases from activating kinase or phosphatase activities (Braley & Piper, 1997). Furthermore, it has been shown that cells without Hsp30 have lower amounts of ATP when under weak acid stress, consistent with increased ATP usage (Piper *et*

al., 1997). A mutant lacking Hsp30 also displayed a longer lag phase (Piper *et al.,* 1997).

The extent at which the H^+-ATPase activity alone could counteract the intracellular acidification as a result of the weak acid stress may be limited, as there is only a finite limit to which its action can enhance the charge part (Z) of the electrochemical potential ($Z\Delta pH$). A possible way for the yeast to overcome this problem is to ensure the movement of charge that compensates for the H^+-ATPase extruded proton. Anion ($XCOO^-$) extrusion or K^+-influx could both satisfy this requirement.

Studies by Piper *et al.* (1998) showed that sorbate adaptation involved the induction of the Pdr12 ABC transporter, a system for ATP-driven extrusion of the acid anion. This benefits the cell in two ways, by lowering intracellular acid levels and balancing the charge movement, aiding a higher level of catalysed proton extrusion (Figure 1.2). Thus the combined actions of the H^+-ATPase and Pdr12 may both be needed to restore homeostasis to the point where the yeasts cells can grow. Both processes are undoubtedly expensive in energy terms and thus would be reflected in dramatic reductions in biomass yield. Such anion and proton expulsion would seem pointless without some system restricting free diffusional entry of the undissociated acid (XCOOH). However, how weak acid diffusion is restricted in adapted cells at present remains unknown (Piper *et al.,* 2001).

The weak acid response is quite distinct from previously characterised stress responses in *S. cerevisiae.* It is regulated by the War1p zinc finger transcription factor and induced by sorbate, benzoate and other moderately lipophilic carboxylate compounds (Piper *et al.*, 1998, Hatzixanthis *et al.*, 2003, Kren *et al.,* 2003 and Schüller *et al.*, 2004). The War1p regulon is small, *PDR12* being the only War1p target gene needed for the pronounced effects of War1p loss on sorbate resistance (Schüller *et al.*, 2004).



Derived from Piper *et al.* (2001).

Figure 1.2

A schematic model based upon Figure 1.1 of how the Pdr12p may help yeast counteract the effects of weak acid stress in yeast. In both Figure 1.1 (unadapted cells) and 1.2 (adapted cells), protonated uncharged acid (XCOOH) is freely permeable across the cell membrane entering the cellular cytoplasm. In weak acid adapted cells, some level of limitation of weak acid entry to the cell must occur (not depicted). The membrane potential difference across the plasma membrane is maintained through the H^+-ATPase-catalysed proton extrusion. The induced Pdr12 aids the adaptation by extrusion of acid anions out of the cell. Induction of both H^+-ATPase and Pdr12 poses problems for homeostasis maintenance in cells adapted to growth in these acids unless there is induction also of a system restricting free diffusional entry of the undissociated acid.

1.6.3.1. Problems from futile cycles and possible changes to the membrane to aid restriction of weak acids

As indicated in Figure 1.2 simply pumping both the H^+ and the acid anions out of the cell could create a futile cycle where the anions re-associate at the lower external pH with the protons into the undissociated form and re-enter the cell. Without such limitation, acid would diffuse into the cells as fast as it is pumped out, resulting in an energetically very expensive cycle. However, studies have shown that acid adapted *S. cerevisiae* and *Z. bailii* can maintain an intracellular verses extracellular distribution of benzoate that is not in equilibrium (Warth, 1977). This indicates that a system must be in place in adapted cells to achieve this. The ability of Pdr12 to give resistance to short chain alkanols, compounds whose toxic effects are thought to be due mainly to their ability to dissolve in membranes (Weber & de Bont, 1996), suggests that this ABC transporter may bind acid anions or alcohol molecules actually incorporated in the inner leaflet of the plasma membrane. One can surmise that Pdr12 then transports these to the opposite (periplasmic) side of the membrane, in order to release them into the aqueous phase of the periplasm. Such active efflux may be able to lower the intracellular level of the acid anion or alcohol, on the basis that the polar groups on these carboxylate anion or alcohol substrates will slow their diffusion back across the cell membrane.

Aims of this study

The aims of this thesis were to study the effects of sorbic acid stress on the physiology of *S. cerevisiae,* hopefully providing further understanding into yeast adaptation to grow in the presence of weak acids.

The first aim of this study was to develop and then use an *in vivo* methodology to investigate the effects of sorbic acid upon intracellular pH in yeast. Other parameters such as membrane permeability and ATP usage were also measured to give further understanding to these stress effects on the cellular physiology of *S. cerevisiae.*

The second aim of the study was to investigate the effects of sorbic acid on the proteomic expression in yeast using a variety of SDS-PAGE techniques. This would hopefully result in the identification of any proteins and hopefully genes that could be important in the cellular response to this stress.

The final aim was initiated as a result of findings from the proteomic study, thus researching the auxotrophic requirements for aromatic amino acids in yeast (in this case for tryptophan) and to understand the reasons for the observed pronounced effect that renders *S. cerevisiae* sensitive to inhibition both by moderately lipophilic weak organic acids such as sorbate, as well as by high levels of the relatively non-lipophilic acetate.

Chapter 2 - Materials and methods

2.1. Yeast Strains

The *Saccharomyces cerevisiae* strains used in this study are detailed in the following subsections below.

2.1.1. Intracellular pH method development studies

RS-514 - *MATa, ade1-100 his4-519 leu2-3, 112 ura3-52, GAL+, Pma1+* (pRS-427)

RS-516 - *MATa, ade1-100 his4-519 leu2-3, 112 ura3-52, GAL+, pma1-205+* (pRS-427)

RS-514 (termed *PMA1* in study), with a fully functional plasma membrane H^+-ATPase, is the isogenic parent of RS-516 (termed *pma1-205* in study), a mutant displaying reduced expression of the plasma membrane H^+-ATPase.
These strains were kindly provided by Ramon Serrano (Departmento de Biotecnologica, Universidad Politecnica, Valencia, Spain).
Both *PMA1* and *pma1-205* were constructed by transforming the strain BWG1-7a with plasmid pRS-427 (Vallejo & Serrano, 1989).

PMA1 was maintained on YPD agar plates (2% w/v glucose (BDH), 2% w/v yeast extract (BetaLab), 1% w/v bacto peptone (Difco) and 2% agar (Difco)). *pma1-205* was maintained on galactose plates (2% w/v galactose (BDH), 0.2% w/v adenine (Sigma), 0.2% w/v histidine (Sigma) and 2% w/v agar (Difco)), to ensure that the *GAL-PMA1* gene on pRS-427 was expressed to assure normal growth.
(Chapter 3.)

2.1.2. Yeast proteomic studies

The parent strain FY1679-28c (*MATa ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63*) was used for the proteomic studies in Chapter 4 and chosen as it was the same parent strain used to produce the deletion mutant library by EUROSCARF. This strain was kindly provided by John Chapman of Unilever Research, Vlaardingen, Netherlands. The strain was maintained on YNBG agar plates (0.67% w/v yeast nitrogen base with amino acids (Difco), 2% w/v/ D-glucose (BDH) and 2% w/v agar).
(Chapter 4).

2.1.3. Phenotype determination studies

The yeast strains used in the phenotype determination study are listed in Table 2.1. The deletion mutant strains were those identified from the proteomic study (Chapter 4) as proteins of interest with an increased expression in response to sorbic acid stress and were purchased from Research Genetics in a BY parental background. All of these strains were maintained upon YPD agar plates, as used for *PMA1* in section 2.1.1. (Chapter 5).

Table 2.1. *S. cerevisiae* strains used in phenotype determination study

Strain	Genotype	Source
FY1679-28c	*MATa ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63*	EUROSCARF
FY1679-11c	*MATa ura3-52 his3-Δ200 leu2-Δ1*	EUROSCARF
BY4741	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0*	Research genetics
BY4743	*MATa/α ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 lys2-Δ0*	Research genetics
ach1Δ	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 Δach1*	Research genetics
hsp26Δ	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 Δhsp26*	Research genetics
cph1Δ	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 Δcph1*	Research genetics
cys4Δ	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 Δcys4*	Research genetics
hyp0Δ	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 Δhyp0*	Research genetics
FY809	*MATa ura3-52 his3-Δ200 leu2-Δ1 Δpdr12::hisG*	Bauer *et al.* (2003)
11c-*azr1Δ*	*MATa ura3-52 his3-Δ200 leu2-Δ1 Δazr1::kanMX4*	Bauer *et al.* (2003)
11c-*pdr12Δ azr1Δ*	*MATa ura3-52 his3-Δ200 leu2-Δ1 Δpdr12::hisG, Δazr1::kanMX4*	Bauer *et al.* (2003)
GAL1-PDR12	*MATa ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63 PDR12::kanMX4-proGAL1*	Hatzixanthis *et al.* (2003)
trp1Δ	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 Δtrp1-kanMX4*	EUROSCARF
trp2Δ	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 Δtrp2-kanMX4*	EUROSCARF
trp3Δ	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 Δtrp3-kanMX4*	EUROSCARF
trp4Δ	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 Δtrp4-kanMX4*	EUROSCARF
trp5Δ	*MATa ura3-0 his3-Δ1 leu2-Δ0 met15-Δ0 Δtrp5-kanMX4*	EUROSCARF
YPH499	*MATa ura3-52 lys2-801^{am} ade2-101^{oc} trp1-Δ63 his3-Δ200 leu2-Δ1*	Bauer *et al.* (2003)
YPH499 TRP^{+}	*YPH499 trp1-Δ63::pRS304(TRP1)*	Bauer *et al.* (2003)
war1-42	*YPH499 war1-42*	Sikorski *et al.* (1989)

(Chapter 5).

2.2. Conditions of yeast culture

2.2.1. Yeast cell starter cultures for intracellular pH probe methodology

The strain *pma1-205* was initially grown in galactose synthetic medium (2% w/v galactose, 0.2% w/v adenine, 0.2% w/v histidine and containing 0.67% w/v yeast nitrogen base with amino acids (Difco)). Flasks were shaken for 48 hours aerobically at 30°C to allow growth. Starter cultures of *pma1-205* were produced by reinoculation into fresh galactose synthetic medium and incubated for further 18 hours at 30°C with shaking.

PMA1 was inoculated straight into YPD media with glucose (2% w/v D-glucose (BDH), 2% w/v yeast extract (Betalab), and 1% w/v bacto peptone. The flasks were incubated exactly the same as for *pma1-205* aerobically at 30°C and shaking, unless otherwise stated.
Overnight cultures for both strains were then used for loading of the fluorescent probe to perform internal pH determinations (see section 2.4.2. for probe loading method). (Chapter 3).

2.2.2. Culture conditions for growth of yeast cells for pH_i determination

Fluorescent probe loaded cells of *PMA1* and *pma1-205* (10 ml at an $O.D._{600}$ of 0.8) were harvested by centrifugation at 2,050 *g* for 10 min (see section 2.4.2. for method). The resultant pellets were then resuspended into 25 ml of YNBG media (0.67% w/v yeast nitrogen base with amino acids, 2% w/v D-glucose) buffered with citric / phosphate (50 mM citric acid, 50 mM sodium di-hydrogen orthophosphate and 50 mM potassium hydroxide (all from BDH)). The pH of this media (termed YNBG-CP) was altered either to pH 4.5 or 3.8.

The cultures were then incubated for 30 min shaking at 30°C to allow the cells to recover from the stress imposed by the probe loading conditions, before being used for intracellular pH determination and growth experiments. (Chapter 3).

2.2.3. Culture conditions for growth of yeast cells for 1D and 2D electrophoresis studies

The strain FY1679-28c was grown in Synthetic Complete (SC) media pH 4.5 (Kaiser *et al.*, 1994 and Panaretou, 1993), which consisted of a completely synthetic media containing all the amino acids and markers required for growth. This media was then prepared with the omission of different amino acids to allow uptake of the chosen radio-labelled amino acid for proteomic studies.

100 ml of SC media was made using, 50 ml of sterile distilled water, 37 ml of complete drop out mix (see below), 10 ml of 20% (pre-sterilised) glucose, 1 ml of each of the four markers (pre-sterilised), 0.4% L-uracil, 0.6% L-histidine, 2.6% L-leucine and 0.8% L-tryptophan (all markers from Sigma).

The complete drop out solution comprised of 3.4 g of YNB without amino acids and ammonium sulphate (Difco), 10 g of ammonium sulphate, and 2.86 g of amino acid mix. This amino acid mix consisted of 0.08 g adenine, 0.04 g argenine, 0.06 g tyrosine, 0.06 g isoleucine, 0.1 g phenylalanine, 0.2 g glutamic acid, 0.2 g aspartic acid, 0.7 g threonine, 0.4 g serine, 0.3 g valine, 0.3 g methionine and 0.36 g lysine (all amino acids from Sigma kit – DAA-20). All of the components were added to 740 ml of distilled water, the pH adjusted to 5.5 and sterilised.

FY1679-28c starter cultures were prepared by inoculation of 100 ml of SC media pH 4.5 and incubated aerobically for 18 hours at 30°C with shaking, to allow growth. A weak acid stress was imposed on some cultures by the addition of 0.9 mM sorbic acid to the media (stock prepared from potassium sorbate - Sigma). (Chapter 4).

2.2.4. Culture conditions for pulse labelling growing yeast cells with ^{35}S methionine for one and two dimensional electrophoresis studies

Overnight starter cultures of FY1679-28c were prepared in 100 ml of SC media (as in section 2.2.3.), but in this case was adjusted to pH 4. The cultures were incubated aerobically for 18 hours at 30°C shaking until a cell number of 8×10^7 cells ml^{-1} was reached. The exponential cultures were then centrifuged at 2,050 *g* for 10 min in a

bench centrifuge (Hettich), washed once and then resuspended in the same media SC media, however, in this instance without the amino acid methionine.

For the pulse label step, two 25 ml cultures resuspended in the above SC media minus methionine were prepared, one with sorbic acid at 0.9 mM and the other a control. 25 µl of the radio-label ^{35}S methionine (Amersham, B42396, 500 µci / 18.5 Bq) was added to both cultures, which gave 250 µci per 25 ml of culture. The cultures were then incubated at 30°C for 1 and 3 hours to allow incorporation of the radio-label into the growing cells. After both of the incubation times, 10 ml samples were removed from each of the flasks and immediately chilled on ice. These samples were then centrifuged at 2,050 *g* for 2 min in a bench centrifuge and the supernatants discarded. The resultant pellets were then rapidly frozen at -80°C until required for electrophoresis studies. (Chapter 4).

2.2.5. Culture conditions for growth of yeast cells for phenotypic determination

All strains listed in Table 2.1 for the phenotypic determination studies were grown overnight using the same culture conditions as those described for *PMA1* in section 2.2.1., however, the pH of the media was adjusted to pH 4.5. (Chapter 5).

2.3. Measurement of growth

2.3.1. Total viable cells agar plate count

The viability of the cells was measured by serially diluting a culture of *S. cerevisiae* with Maximum Recovery Diluent (MRD, from Biomerieux). 0.1 ml volumes of each dilution were spread onto YPD agar plates, incubated at 30°C for 24 hours and the resultant colonies counted. (Chapters 3 and 5).

2.3.2. Specific growth rate determination using optical density measurements

Optical densities of growing cultures of *S. cerevisiae* were measured using a spectrophotometer (Philips PU 8630). 1 ml culture volumes were added to 1.6 ml semi-micro plastic cuvettes (Alpha Labs – W1) and measured using a wavelength setting of 600 nm ($O.D._{600}$). The resultant optical density measurements were plotted ona logarithmic scale ($\log_{10}$) and the specific growth rates calculated from the resultant line gradients then multiplied by a factor of 2.3. (Chapters 3 and 4).

2.3.3. Specific growth rate determination using automated Bioscreen analyser

Optical densities of growing cultures of numerous strains of *S. cerevisiae* in the presence of varying stress agents could be measured simultaneously using a Labsytems Bioscreen automated turbidometric analyser (Life Sciences International, Basingstoke, UK).

Overnight cultures as prepared in section 2.2.5. were diluted using YPD pH 4.5 to an $O.D._{600}$ of 0.2. 20 µl of each strain was added per well of a Bioscreen microtitre plate (100 well honeycomb, Life Sciences International) to give an inoculum size of 5.0 x 10^3 $cellsml^{-1}$. Dilution ranges of inhibitory agents were prepared using YPD media at pH 4.5 and 380 µl of these agents were added per well, in triplicate for each dilution per yeast strain. Growth at 30°C with continuous shaking was monitored hourly, measuring changes in the $O.D._{600}$ using the automated Bioscreen analyser. As in the previous section 2.3.2 the optical density readings were plotted on a logarithmic scale to compare and calculate the specifc growth rates for the strains tested.
(Chapter 5).

2.3.4. Growth inhibition determination using phase inhibition agar plates inoculated with stress agents

YPD agar plates (pH 5.6) were prepared and allowed to cool at room temperature. A 10 mm hole was cut into the centre of the agar for each test plate, the agar plug removed and each plate then divided into 5 sectors. 100 µl volumes of either plain media or sorbic acid at a range of concentrations were added to the holes in the agar plates. The plates were inoculated with each of the test strains, incubated at 30°C for 24 hours and amount of growth observed.

Alternatively YPD agar at pH 4.5 containing either sorbic acid or acetic acid at tests levels indicated were used to test the growth of the various mutant strains. Overnight cultures of each of the test strains were added to the plates at neat, 1 in 10 and 1 in 100 serial dilutions; these were pinned onto solid YPD agar and incubated at 30°C for 3 days (Bauer *et al.*, 2003). (Chapter 5).

2.4. Methods for intracellular pH measurement

2.4.1. Intracellular pH calibration curves

Calibration curves were constructed using a pH dependent fluorescent probe 5'(6') carboxy fluorescein diacetate succinimidyl ester (CFDA-SE from Lambda Fluorescence Technologie, Graz, Austria).

To cleave the non fluorescent CFDA-SE into the fluorescent carboxy fluorescein (CF-SE) form, 30 $\mu g ml^{-1}$ CFDA-SE was made up from a stock solution in dimethyl sulphoxide (Sigma) and 50 $\mu g ml^{-1}$ of porcine esterase (Sigma) was added to 1 ml of distilled water. The solution was vortex mixed and then incubated at ambient temperature for 12 hours in the dark to allow probe cleavage.

The calibration curves were then constructed using the cleaved probe (CF-SE) and added to YNBG-CP medium containing permeabilised yeast cells over a range of pH values (from pH 7.0 to 4.5 in increments of 0.2 of a pH unit). Permeabilised cells were prepared from an exponential yeast culture of *PMA1* (grown overnight in

YNBG at 30°C), diluted to approximately an $O.D._{600}$ of 0.8 and then exposed to 4.37 µM amphotericin B (Sigma) for 60 min at 30°C. The cells were then centrifuged and then resuspended to the same concentration into fresh YNBG-CP medium (at the various pH values stated above). Each pH calibration point was prepared by adding 30 µl of the cleaved CF-SE probe to 1.5 ml of YNBG-CP medium plus cells at the various pH values. Each sample was vortex mixed and then added to a quartz cuvette to measure the fluorescence level for each pH value.

Fluorescence determinations were made in a Shimadzu RF-1501 fluorimeter (Shimadzu UK, Haverhill, Suffolk) using a 1.5 ml optically clear quartz cuvette (Helma, Fisher Scientific UK). The cleaved CF-SE probe was measured using an excitation scan between 400 nm and 500 nm with the emission set at 525 nm (bandwidths 10 nm).

Calibration curves were constructed by plotting the ratio of fluorescence intensities (emission wavelength 525 nm) at the excitation wavelengths of 495 nm (pH-dependent point) and 435 nm (pH-independent point) as a function of pH. The intracellular pH could then be calculated from this calibration curve.
(Chapter 3).

2.4.2. Optimisation of loading *S. cerevisiae* cells with the CFDA-SE probe

A matrix comprising of buffer pH, CFDA-SE concentration, loading temperature and duration of loading incubation was set up to identify optimal conditions for probe loading whilst maintaining cell viability.

Cell cultures of *PMA1* were grown overnight at 30°C in YNBG-CP, diluted to an $O.D._{600}$ of approximately 0.8 and then centrifuged at 2,050 *g* for 10 min. The pellets were resuspended in an equal volume of 100 mM citric / phosphate or CP buffer (citric acid and sodium di-hydrogen orthophosphate) at pH 4.0 or 6.5. The CFDA-SE was added at two concentrations, 50 and 100 µM, and the cells were incubated at 30, 35, 37 and 40°C (using water-baths) for 0, 7, 12 and 24 hour periods.

Following these different loading regimes, the cell viability was measured by using a total viable count on YPD agar (as described in section 2.3.1.) and growth rates compared to unloaded cells by resuspension in YPD broth (5 x 10^3cellsml^{-1}) at pH 5.7 and growth was measured at $O.D._{600}$ (section 2.3.2.). Comparison of the total level of fluorescence of the loaded cells was determined in the fluorimeter as described above in section 2.4.1. (Chapter 3).

2.4.3. Confirmation of fluorescent probe loading using CSLM

To confirm the level of probe loaded within the cell culture and distribution of the loaded probe within the cytosol of individual cells, loaded cells were studied using a confocal scanning laser microscope (CSLM). The cells were visualised using a Bio-Rad MRC 600 CSLM fitted with a 20 mW Krypton Argon mixed gas laser (Bio-Rad) and with an objective magnification of x 60 (Nikon x 60 oil, 1.4 numerical aperture and Plan Apo objective). Split screen images were acquired using a dual-channel collection mode. The first channel was a transmitted illumination phase contrast image and the second channel was an epi-fluorescent image of the intracellular CF-SE (excitation line 488 nm). Each image was averaged over at least three frames to reduce background noise.
(Chapter 3).

2.4.4. Determination of intracellular pH (pH_i)

Yeast cells as described in sections 2.2.2. and 2.4.2. were grown aerobically to mid-exponential phase in YNBG-CP at 30°C with shaking. These cells were loaded with the fluorescent CFDA-SE probe at the optimised conditions of 100 mM citric / phosphate buffer at pH 4.0 containing 100 µM CFDA-SE. The cells were then incubated at 37°C for 24 hours, to allow loading of the fluorescent probe.
To demonstrate the effectiveness of this *in vivo* technique to measure rapid changes in pH_i, 5.5 mM sorbic acid was added to loaded cultures of *PMA1* in citric / phosphate buffer at pH 3.4 after approximately 30 min at 30°C. 2.78 ml of a pre-warmed sorbic acid at a stock concentration of 50 mM in distilled water was added to a 25 ml culture volume. 1.5 ml samples were continued to be removed for the

duration of the experiment from all test flasks at regular time intervals to measure both growth (section 2.3.2.) and intracellular fluorescence (section 2.4.1.).

To ensure that leakage of the probe from the loaded cells was not resulting in background fluorescence, it was necessary to measure levels of fluorescence in the culture supernatant alone. Therefore, for each sample taken, the $O.D._{600}$ measurement was performed first (if growth was being measured) and then a total fluorescence reading was determined followed by a fluorescence reading of the supernatant. For the latter, the sample was centrifuged at 23,430 *g* for 4 min in a bench centrifuge and the supernatant removed from the cell pellet. Any background fluorescence detected in this sample was then subtracted from the total fluorescence reading.

In a second experiment the pH_i of both *PMA1* and *pma1-205* were measured in citric / phosphate buffer at pH 4.5. Again after 30 min at 30°C, 5.5 mM sorbic acid was added to the culture flasks and the pH_i continued to be measured for the duration of the experiment. In a third experiment, both pH_i and growth were simultaneously measured of the fluorescent loaded cells of *PMA1* and *pma1-205,* both were resuspended into YNBG-CP media at pH 3.8, without the addition of any sorbic acid. (Chapter 3).

2.4.5. Effect of antifungal compounds on the pH_i of yeast cells

For experiments comparing the cellular effect of sorbic acid and amphotericin B upon the pH_i of growing yeast cells, the following protocol was followed.
CFDA-SE loaded cells were centrifuged 2,050 *g* for 10 min and resuspended in 25 ml of YNBG-CP at either pH 4.5 or 3.8 to give a starting $O.D._{600}$ of approximately 0.4. After a 30 min recovery at 30°C, both growth and pH_i were measured at regular intervals as described in section 2.4.4.

When cultures had grown to an $O.D._{600}$ of approximately 0.55, sorbic acid at a range of concentrations (0 to 1.6 mM undissociated sorbic acid) and amphotericin B (from 0 to 9.5 µM, using a 0.5 mM stock solution) were added. The amphotericin B was initially solubilised in dimethyl sulphoxide (both from Sigma) to a concentration of 10 mM and from this stock, the working 0.5 mM solution was made in distilled

water. Following these additions, growth and pH_i of the samples were measured for a further time period.

In another set of experiments, the same concentration of undissociated sorbic acid (0.22 mM) and 0.35 µM amphotericin B were added to cultures at different pH values (3.2, 3.8, 4.2, 4.5 and 5.0). The total concentration of sorbic acid was calculated assuming the pKa of sorbic acid to be 4.74 using the Henderson-Hasselbalch equation. The change in pH_i due to the addition of sorbic acid and amphotericin B was calculated as the difference between the pH_i prior to the addition of each compound and the final determination of pH_i after the addition of each compound. The final determination was made 4 hours after the addition of each compound when the pH_i value had remained stable for at least 1 hour.
(Chapter 3).

2.5. Determination of yeast membrane permeability in the presence of antimicrobial compounds

Cells were grown in YNBG at 30°C with shaking, harvested using centrifugation (2,590 *g* for 10 min, 4°C), washed in distilled water and resuspended in YNB, without glucose, at pH 5.0, 4.2 and 3.2 to a final cell density of 35 mg wet weight ml^{-1}. These cells were then exposed to either, 0.22 mM undissociated sorbic acid, or 0.35 µM amphotericin B for 10 min at 30°C, and again harvested, washed and resuspended in sterile distilled water. The change in the external pH of the cell suspension (net proton flux) was determined at either pH 5.0, 4.2 or 3.2 (adjusted with 1 M hydrochloric acid or 1M sodium hydroxide – from BDH) for 5 min at 30°C (as previously described by Coote *et al.*, 1994). Results were expressed as the decrease in extracellular H^+ ion concentration $mg^{-1}ml^{-1}$ wet weight cells.
(Chapter 3).

2.6. Method for measuring intracellular ATP and ADP levels in yeast

This method was an adaptation from that described by Chapman *et al.* (1971).

Cells were inoculated into YNBG-CP, pH 3.2, at 30°C with shaking to give a starting $O.D._{600}$ of approximately 0.4. When the cultures had grown to an $O.D._{600}$ of approximately 0.55, 0.22 mM sorbic acid (undissociated acid) and 0.35 µM amphotericin B were added. Growth plus ATP and ADP levels were measured pre and post-addition of sorbic acid and amphotericin B.

For the determination of intracellular ATP and ADP levels the Celsis High Sensitivity Bioluminescence Kit was used (Celsis International, Cambridge Science Park, Cambridge, UK). For each culture, two 200 µl samples of cells were each added to 200 µl of ATP releasing agent (Celsis) in sterile eppendorfs, mixed for 10 s, and then frozen in liquid nitrogen before storage at -80°C. At a later date samples were thawed rapidly by centrifugation at 17,300 *g* for 20 min at room temperature.

Intracellular ATP levels were measured by adding 150 µl of supernatant from the first eppendorf and added to 75 µl of phosphate buffer at pH 7.3 (75 mM potassium di-hydrogen orthophosphate / di-potassium hydrogen orthophosphate, 15 mM magnesium chloride and 0.5 mM phospho-enol pyruvate, all from Sigma). This was vortex mixed and incubated for 15 min at 25°C, before a 5 µl sample was transferred to a Microlite-1 luminescent assay micro-titre plate (Dynatech Laboratories Inc., Chantilly, Virginia). 60 µl of bioluminescence reagent (containing firefly luciferase from Celsis) was then added and light emission was measured after a 1 s delay over an integration period of 10 s using a Luminoscan luminometer (Labsystems, Basingstoke, Hampshire, UK).

To measure intracellular ADP levels, the same procedure was carried out using the second eppendorf, except 20 µl of pyruvate kinase at 1 $mgml^{-1}$ (Sigma) was added to each sample to convert the ADP to ATP. To measure the extracellular ATP and ADP, the cells were removed from the culture samples by centrifugation (17,300 *g* for 4 min), the supernatant was retained and ATP and ADP levels measured as above. (Chapter 3).

2.7. Sample preparation of *S. cerevisiae* for separation by electrophoresis

2.7.1. Preparation of total protein preparations from *S. cerevisiae*

This method was an adaptation of that described by Panaretou & Piper (1992) and Cash (1991).

Two 400 ml overnight cultures of *S. cerevisiae* FY1679-28c strain of both a control and sorbic acid stressed (using 0.9 mM) were prepared as described in section 2.2.3. and 2.2.4. The optical densities were measured at $O.D._{600}$ and noted to be approximately 0.8.

The cultures were centrifuged at 3,024 *g* for 10 min at 10°C in an ultra-centrifuge (Beckman Avanti – rotor JA) and the supernatants removed. The pellets for each culture set were resuspended into 100 ml of chilled 20 mM trizma hydrochloride (Tris / HCl - Sigma) at pH 7. The pellets were then resuspended by vortex mixing and centrifuged again at 5,580 *g* for 10 min at ambient using a bench top centrifuge. The centrifugation and resuspension step was repeated once more for each culture set. The resulting supernatants were decanted and discarded, and the pellets resuspended with 1.5 ml of lysis buffer.

The Lysis buffer was prepared using 13.5 g urea (Sigma ACS Reagent), 0.5 ml triton X-100 (Sigma Ultra), 0.5 ml 2-mercaptoethanol (Sigma), 0.5 ml pharmalyte 3-10 (Pharmacia), 35 mg phenylmethanesulfonyl fluoride (PMSF- Sigma) and 20 µl of 2.5 $mgml^{-1}$ pepstatin A (Sigma) which had been presolubilsed into methanol (Sigma). The urea was added first, to 10 ml of the distilled water and allowed to dissolve by heating the contents using a hot water bath (80°C). The remainder of chemicals were added to this solution and the volume made up to 25 ml with distilled water. The protease inhibitors PMSF and pepstatin A were added just before use and prepared fresh each time.

Each resuspended pellet was added to a large clean glass boiling tube and to this a spatula spoon full of large 20 mm glass beads and a spatula spoon full of smaller 400 µm glass beads (both Pierce and Warriner) were added. To each tube 2 to 3 drops of Antifoam (Sigma – pre-autoclaved) was then added and the tops of the tubes covered with Parafilm. Each tube was then vortexed for 1 min and then allowed to rest for 1 min on ice, this was repeated 10 times to ensure complete break up of the yeast cells. After this vortex sequence the tubes were left on ice for 20 min to allow the contents of each to settle.

The supernatant from each tube was removed and added to sterile eppendorf tubes. The contents of each were centrifuged at 2,050 *g* in a bench top centrifuge for 4 min at 4°C. The resulting supernatants were removed and added to fresh sterile eppendorf tubes. To each solution (approx. 2-3 ml) 250 µl of a 50% w/v glycerol solution in distilled water (made from a 100% stock - Sigma) was added and the samples were then aliquoted into 250 µl lots before being stored at -80°C.
(Chapter 4).

2.7.2. Extraction of total protein preparations into Laemmli sample buffer

To precipitate the protein out from its existing solution, the protein samples prepared in section 2.7.1. were defrosted and 100 µl added of each to sterile eppendorf tubes. To these 500 µl of acetone (BDH which was chilled at -20°C for 1 hour prior to use) was added and each sample mixed by vortex mixer for 5 to 10 min. Once all of the protein had precipitated out of solution, the samples were centrifuged at 23,430 *g* in a bench centrifuge for 10 min. The supernatants were then removed from each pellet and the tubes left for 30 min with the lids open at room temperature to allow the remaining acetone to evaporate off.

Laemmli sample buffer (Laemmli, 1970) was prepared by adding 2.5 ml of 0.5 M Tris / HCl pH 6.8, 2.0 ml of 10% w/v lauryl sulphate sodium salt (SDS), 2.5 ml of 40% w/v glycerol, 0.5 ml of *B*-mercaptoethanol, 0.3 ml of 1% w/v bromophenol blue in distilled water (all from Sigma) and 2.2 ml of distilled water, making a total volume of 10 ml. This was stored at 4°C until required.

The pellets were then resuspended into 1 ml each of Laemmli sample buffer and vortex mixed until dissolved. An increased sample volume was used to allow full dissolving, and thus equalled a 1 in 10 dilution of the original sample. The samples were then stored at -80°C till required. (Chapter 4).

2.7.3. Estimation of protein concentration of total protein preparations using Biorad assay

A protein standard curve was constructed using the Biorad protein standard (Biorad – from a stock concentration of 1.38 $mgml^{-1}$). The standard was diluted to a range of concentrations; 0, 1, 5, 10, 15, 20 and 25 μgml^{-1} in distilled water. Using sterile eppendorfs, 800 µl of each standard was added to 200 µl of Biorad protein assay reagent (Biorad), each sample was then vortex mixed. 1 ml of each sample was then pipetted into clean plastic cuvettes and the optical density of each sample measured at an $O.D._{595}$. For a blank sample the 0 μgml^{-1} was used.

The protein samples that had been precipitated into Laemmli sample buffer (see section 2.7.2.) were diluted 1 in 100 in this buffer and then measured in the above Biorad assay, determining the concentration of protein present in both the control and sorbic acid samples. A calibration curve was then constructed to calculate the protein concentration of the control and sorbic acid total protein preparations.
(Chapter 4).

2.7.4. Estimation of the level of radio-label incorporated into the pulse labelled protein samples using scintillation counting

Both ^{35}S pulse labelled control and sorbic acid samples dissolved in Laemmli sample buffer (see section 2.7.2.) were defrosted and 20 µl of each sample were added in duplicate to sterile eppendorfs. 5 ml of a chilled 5% w/v trichloroacetic acid (TCA - Sigma) and 0.1% w/v bacto peptone solution in distilled water was added to each sample, which was then vortex mixed and incubated on ice for 15 min, to allow precipitation.

Using 2.5 cm diameter filter papers (Whatman GF/C) each precipitated sample was filtered through the paper and then washed using one lot of 5 ml of TCA/ bacto peptone solution and then two lots of 5 ml ice cold absolute ethanol (BDH). Each sample filter paper was dried at 120°C for 15 min and then the sample including the filter paper placed in a 5 ml scintillation counting vial. To each vial, 16 ml of Ready Safe scintillation cocktail fluid (Beckman) was added and then all of the vials were read in a Beckman scintillation counter using a ^{35}S standard and settings. The samples were measured calculating the amount of disintegrations per minute (DPM) and then converted into counts per min in order to calculate the amount of radioactive sample to add to each gel. (Chapter 4).

2.8. Method for one-dimensional gel separation of total protein preparations of *S. cerevisiae*

2.8.1. One-dimensional gel electrophoresis using standard *S. cerevisiae* cultures

The total protein preparations prepared in section 2.7.2. were separated using one dimensional gel electrophoresis. The method below is the adaptation of that described by Rabilloud (ed.) 2000 and the mini gel electrophoresis kit was constructed and prepared as described by reference.

The first gel layer, a 12% resolving gel consisted of 200 µl of 10% w/v SDS in distilled water, 6 ml of 40% v/v acrylamide / bis-acrylamide (Sigma), 5 ml of 1.5 M Tris / HCl pH 8.8 and 8.7 ml of distilled water. These reagents were mixed together carefully so not to include any air. Two other reagents were then added, 200 µl of 10% w/v ammonium persulphate (Sigma) in distilled water and 20 µl of N,N,N',N'-Tetramethylethylenediamine (TEMED - Sigma) and the solution was gently mixed again. Using a sterile disposable pipette, the gel was carefully added between the plates, ensuring no bubbles were incorporated and added up to the marked line. A total volume of 10 ml of resolving gel was required. The gel was allowed to set for 30 min at room temperature, with a layer of distilled water added to the top which excluded air from the gel whilst setting.

The second gel consisted of a 4% stacking gel strength, and 5 ml of gel was prepared by adding 0.5 ml of 40% v/v acrylamide, 1.26 ml of 0.5 M Tris / HCl pH 6.8, 50 µl of 10% w/v SDS and 3.18 ml of distilled water. These reagents were mixed gently together before 10 µl of TEMED and 50 µl of 10% w/v ammonium persulphate were added and the gel was mixed gently again.

Once the first resolving gel had set, the distilled water was poured off and the second stacking gel added to a depth of 2 cm. The sample well comb was inserted into this gel, ensuring no bubbles were caught underneath the teeth. The gel was allowed to set for a further 30 min at room temperature.

A 10 times concentrate of running buffer for the gel was prepared and then diluted to one times working strength as needed. The concentrate consisted of 250 mM Trizma Base (Sigma), 1.92 M glycine (Sigma) and 1% w/v SDS and the pH adjusted to 8.3. For each run, 250 ml of one times strength running buffer was made fresh each time.

The mini gel contained a maximum of 10 wells, of which 2 were used for molecular weight markers, and 8 for samples. Two different total protein preparations were added to the gel, a control culture and the other a sorbic acid stressed culture (see sections 2.7.1. and 2.7.2 for preparation details). Dilutions of neat, 1 in 1.5, 1 in 2 and 1 in 3 were prepared in sterile eppendorfs using Laemmli sample buffer to give 25 µl volumes of each sample. To the 8 samples, 5 µl of a mixture of bromophenol blue stain and 50% w/v glycine (prepared by adding a few grains of the stain to the glycine in distilled water) was added to aid tracking of the samples through the gel. The samples were then stored on ice until required.

Using long tipped sterile pipettes for each sample (Gilson), 30 µl of sample was added to each well and the molecular weight low range markers (Biorad - 78900) were added to each of the end wells, adding 2 µl per well.

The gel housing was then assembled and connected up to a 1000/500 variable power pack, setting the power output to 650 volts / 35 mAmps. The gel was run until the dye front reached almost the bottom of the gel and then the power was switched off.

The resultant gel was then stained. The gel was firstly soaked in 100 ml of distilled water for 20 min at room temperature to remove any residual SDS and then added to 100 ml Gelcode (Pierce and Warriner) and stained for 1 hour at room temperature, agitating the stain and gel occasionally. Lastly the gel was added to 100 ml of distilled water until the separated bands were clear and the staining enhanced. The gel was then dried on blotting paper, covered with cling film and placed in the vacuum drier for 20 min. (Chapter 4).

2.8.2. One-dimensional gel electrophoresis using radio-labelled ^{35}S methionine *S. cerevisiae* cultures

Radio-labelled control and sorbic acid stressed yeast cultures as prepared in sections 2.7.1. and 2.7.2. were separated using the one-dimensional electrophoresis method as described in section 2.8.1.

However, the gels were stained using Gelcode and were then exposed to autoradiography film – Hyperfilm MP (Amersham) to analyse the radioactive levels in the bands. The gels were placed onto the camera film and exposed to the film within an autoradiography cassette (Hypercassette – Amersham) at room temperature for 10 min to allow high resolution spots to develop. The films were then incubated in developer (Kodak – first developer, E6) solution for 2 min, rinsed in water and the placed in fixative (Kodak – final rinse, E6) for a further 2 min. Finally the films were washed in water for 10 min before being allowed to air dry.
(Chapter 4).

2.9. Method for 2D-PAGE separation of total protein preparations of *S. cerevisiae*

Methods for both the first and second dimensions of the electrophoresis separation were used as described by Pennington & Dunn (2001).

2.9.1. Preparation of the first dimension Immobiline dry strips

Immobiline DryStrips (Pharmacia) were used with a range of pH 4 - 7 and 18 cm in length. The protective backing plastic was removed before adding the gel layer face down into the slot, the point of the strip facing downwards. 350 µl of rehydration solution was added per strip and comprised of 8 M urea (Sigma), 0.5% v/v triton X-100, 50 mg of dithiothreitol (DTT - Pharmacia), 0.13 ml of pharmalyte (Sigma), a few grains of bromophenol blue and made up to 25 ml with distilled water. 2 to 3 ml of DryStrip Cover fluid (Pharmacia) overlaid the strips to prevent evaporation and urea crystallisation. The strips were then left for a minimum of 10 hours to rehydrate at room temperature. Once rehydrated, the strips were rinsed in distilled water and any excess moisture was absorbed using blotting paper.
(Chapter 4).

2.9.2. First and second dimension separation of the total protein preparations

The total protein preparations in lysis buffer from section 2.7.1. were defrosted and diluted in the same solution to ensure similar amounts of protein were loaded onto the gel strips.

The Multiphor 2 (Amersham Pharmacia Biotech) was used to perform the two dimensional electrophoresis. For the first dimension, the temperature was set to 20°C and the kit assembled according to the manufacturers' hand-book. The rehydrated Immobiline strips were placed into the grooved liner gel side up, the acidic (pointed) end placed at the top of the tray and the neutral end at the bottom. 100 µl of diluted sample was then added to each of the sample cups in order to load into the gel strips. The kit was assembled and run for 16 hours using program 8 in the user manual.
Once the strips had run they were stored in foil and frozen until required for the second dimension run, as only one strip could be run at any one time.

The loaded Immobiline strips were equilibrated before the second dimension separation. Two different equilibration solutions (A and B) were used for this step. The base equilibrium solution comprised of 20 ml of 0.38 M Tris /HCl pH 6.8, 72 g of urea, 60 ml of 100% glycerol, 2g of SDS, adding distilled water to make a final

volume of 200 ml. Solution A was further prepared by adding 125 mg of DTT to 25 ml of basic equilibrium solution. Solution B was prepared by adding 1.125 g of iodoacetamide (Sigma) and a few grains of bromophenol blue to 25 ml of equilibrium solution.

The Immobiline strip was incubated first in 20 ml of solution A for 10 min at room temperature and the dish agitated to ensure full mixing. After the incubation, the excess solution was absorbed from the strip on blotting paper and then incubated in 20 ml of solution B for a further 10 min.

The Multiphor electrophoresis kit temperature was reset to 15°C and an ExcelGel SDS two-dimensional precast gel (Pharmacia Biotech, 12-14, 17-1236-01, 245 x 180mm) was added. The gel orientation was noted by cutting the corner off the right hand lower side. ExcelGel SDS Buffer strips (Pharmacia Biotech) were used, anodic (positive) placed on the anodic side of the gel, and similarly cathodic strips (negative) on the cathode side. The equilibrated first dimension immobiline strip was placed 1 mm from the cathode buffer strip, orientating the strip point facing downwards. The gel was then run according to the manufacturers' instructions.

The gel was stained using Gelcode stain as for the one dimensional separation described in section 2.8.1. Photographs were taken of the stained separated proteins within the gels and then electronically scanned to provide digital images for computer analysis. (Chapter 4).

2.9.3. Separation of radioactive ^{35}S total protein samples using two-dimensional electrophoresis

The methods as described in sections 2.9.1. and 2.9.2 for the first and second dimensions were used for the separation of the radioactive control and sorbic acid stressed samples, but with the following amendments.

The same amount of radioactive counts per sample was added to each gel so that the spot sizes for the preparations created on the autoradiographs could be compared. The data from the scintillation counts was used to dilute the control and sorbic acid

samples to ensure this. In addition, samples containing the same amount of protein were also added to gels for all the preparations, the results from the Biorad assay were used to determine the sample dilutions required to provide this. The spots size for each of the separated proteins could then be compared on the stained gels.

The autoradiograph films were prepared by exposing all of the gels to autoradiography film – Hyperfilm MP within an autoradiography cassette. The cassettes were incubated at -80°C for 2 weeks to allow high resolution spots to develop. After this time the cassettes were defrosted for 2 hours, the films removed and allowed to reach room temperature. The films were developed as previously described in section 2.8.2. (Chapter 4).

2.10. Computer analysis on the two-dimensional gel separations

Using a 2D Elite software imaging package (Amersham Pharmacia Biotech, Image Master 2D Elite) all of the two-dimensional gels and corresponding autoradiographs were digitally scanned using a Sharp Scanner (LabScan JX330) and transferred into the software package for analysis.

The software package was then used to compare all of the gels for a set experiment which measured the pixel intensity for each protein spot. To determine if there were any significant changes in levels of expression due to the stress a control gel was compared to a sorbic acid stressed sample. Only if these changes were observed numerous times were the proteins excised from the master gel set and analysed by mass spectrometry. (Chapter 4).

2.11. Identification of spots by molecular fingerprinting by mass spectrometry

The methods used protein excision from the 2D gel, peptide mass fingerprinting of the excised spots and database searching, were described by de Nobel *et al.* (2001). (Chapter 4).

Chapter 3 – The development of an *in vivo* method for measuring intracellular pH (pH_i) and investigating the effect of antifungal compounds on the pH_i of yeast cells

3.1. Introduction

3.1.1. Role of pH_i in the growing yeast cells

Maintenance of pH_i homeostasis within a narrow range is believed to be critical to the correct functioning of the microbial cell (Booth & Kroll, 1989). Despite low external pH, yeast cells are able to maintain their pH_i around neutrality (Kotyk, 1963) in order to sustain optimal activity of major metabolic pathways (Busa & Nuccitelli, 1984). Yeast cells use a dedicated enzyme for maintenance of pH_i homeostasis, the proton-translocating plasma membrane H^+-ATPase. Perhaps indicating the importance of pH_i homeostasis to the cell, this enzyme is essential for growth (Serrano *et al.*, 1986) and can consume up to 60% of total cellular ATP (Serrano, 1991). One of its main functions in consuming this ATP is to generate a proton gradient across the cell membrane for the active transport of nutrients (Eddy, 1982 and Serrano, 1984). This enzyme has also been shown to be involved in the mechanisms of resistance to heat (Coote *et al.,* 1994) and weak-acid preservative stress (Holyoak *et al.*, 1996).

Minor fluctuations in pH_i are believed to play a regulatory role by mediating many important cellular processes (reviewed in Busa & Nuccitelli, 1984). For example, changes in pH_i may play a regulatory role in the cell cycle (Anand & Prasad, 1989) with the rates of DNA and RNA synthesis increasing as the pH_i increases over a narrow physiological range (Madshus, 1988). A reduction in pH_i has been suggested to be a potential trigger for the heat shock response (Coote *et al.*, 1991 and Weitzel *et al.*, 1987). In addition, key glycolytic enzymes are believed to be regulated by pH_i, particularly phosphofructokinase, which is an important flux-controlling enzyme in the glycolysis pathway (Francois *et al.*, 1986). A primary mode of action of weak-acid preservatives is to reduce pH_i below the normal physiological range, leading to growth arrest (Krebs *et al.*, 1983 and Cole & Keenan, 1987) and a key point of interest with regard to this thesis.

3.1.2. Methods for determining pH_i in *S. cerevisiae*

Several approaches have been described for determining pH_i in *S. cerevisiae*:

- One of the most widely used methods is the equilibrium distribution of weak acids (Kotyk, 1963). This has limited application as an *in vivo* method because it requires pH_i measurements to be made using cells resuspended into buffer following various centrifugation steps. Potential difficulties with this method include the inhibitory effects of weak acids on the cell physiology, although Kotyk (1963) reports low cell toxicity with his dye choice. Other issues such as compartmentalisation of weak acids within the cell and the estimation of changes in cell volume which have been shown to alter significantly upon environmental change (Cole & Keenan, 1986), thus causing possible errors in the pH_i data.

- Another widely used method is ^{31}P nuclear magnetic resonance spectroscopy (Gillies *et al.*, 1981 and Coote *et al.*, 1994). Although non-invasive, a universal problem with this technique is the need to use high cell densities leading to an indeterminate physiology of the cells. The technique also suffers from limited time resolution and the need for complex, expensive equipment, as well as a skilled operator (Slavik, 1982).

- Measurement of pH_i in individual cells can be carried out using pH-sensitive microelectrodes, which are implanted within in the cell. Numerous researchers have commented on their use, but conclude that they were not successful in their application (Kotyk, 1963 and Smith & Raven, 1979). Smith & Raven (1979) discuss further their extensive use to measure intracellular pH in larger plant and animal cells but state such electrodes are destructive and are thus clearly not suitable for studying large populations of organisms.

- Many studies have used pH-dependent fluorescent probes, or dyes, to measure pH_i in yeast (Slavik, 1982, and Slavik & Kotyk, 1984) and bacteria (Molenaar *et al.*, 1991). Membrane permeable prefluorochromes, such as fluorescein diacetate (FDA) and 5 (6-)-carboxyfluorescein diacetate (CFDA),

can be loaded into the cytosol, where they are hydrolysed by intracellular esterases into their polar forms whose fluorescence is pH dependent (CFDA is hydrolysed to the fluorescent form carboxy fluorescein (CF)). This pH dependant fluorescence then forms the basis of such methods to determine pH_i. Fluorescent probes such as CFDA have the potential advantage of being rapid, inexpensive, and non-invasive way of determining pH_i under truly *in vivo* conditions. For example, two independent studies have successfully used 5(6)-carboxyfluorescein diacetate (CFDA) (Imai *et al.,* 1994) and 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (Cimprich *et al.*, 1995) with a fluorescence microscope image processing technique to study pH_i in *S. cerevisiae.*

Until recently a fundamental drawback of fluorescent techniques using such probes as FDA and CFDA, was the efflux of the cleaved probe from the cells back into the external environment (Haughland, 1996). This leads to high background levels of fluorescence and thus potentially erroneous readings. Breeuwer *et al.* (1996) used a novel derivative of CFDA termed 5(6-)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) to determine pH_i in the bacteria *Lactococcus lactis, Listeria innocua* and *Bacillus subtilis*. Upon entry into the cytosol, cellular esterases cleave CFDA-SE to the fluorescent form carboxy fluorescein succinimidyl ester (CF-SE). This succinimidyl group is believed to form conjugates with the aliphatic amines on proteins and other intracellular molecules in the cell (Haughland, 1996 and Weston & Parish, 1990), which results in an increased amount of probe retained within the cell and reduces significantly the problem of efflux of the probe back into the external environment (Breeuwer *et al.*, 1996). As suggested by Breeuwer *et al.* (1996), this use of a conjugated probe allows for the measurement of pH_i under conditions that might lead to damage to cell membranes and would otherwise lead to the complete leakage of the probe from the cells. In this Chapter the application and adaptation of this method is developed further for the measurement of pH_i in growing cells of *S. cerevisiae.*

3.1.3. The effect of the sorbic acid on pH_i

The antimicrobial action of organic acids, such as sorbic acid, increases with decreasing culture pH (Booth & Kroll, 1989), and since a greater proportion of undissociated molecules exist at lower pH values, it has been assumed that the undissociated acid is responsible for the antimicrobial effect. However, this does not account for any inhibitory effects the acid may have inside the cell.

Yeast cells are known to maintain their pH_i within a narrow physiological range (between approximately 5.0 and 6.5) despite large decreases in external pH (Cimprich *et al.,* 1995, and Imai & Ohno, 1995). Therefore, because sorbic acid has a pKa of approximately 4.76, at a higher external pH the uncharged undissociated acid diffuses readily across the cell membrane to dissociate almost completely upon entry into the cell, resulting in the release of protons and anions. The acid anion will tend to accumulate intracellularly to very high levels, as being charged it cannot readily diffuse from the cell. This high anion accumulation may generate abnormally high turgor pressure and can also influence free radical production, leading to oxidative stress (Piper *et al.*, 2001).

As a consequence of these findings and many other previous studies, some researchers have proposed that a reduction in pH_i and/ or accumulation of toxic levels of anion, are responsible for growth inhibition (Salmond *et al.,* 1984 and Cole & Keenan, 1987). It is improbable that this model represents the complete explanation and that the anion and acid have numerous effects on a multitude of cellular processes, or that all acids inhibit identically (Piper *et al.*, 2001). For example, acetate and sorbate, with identical pKa's (4.76), have very different inhibitory activity (Salmond *et al.,* 1984). Whilst acetate may be acting as a typical weak acid, the more hydrophobic sorbate could be both inhibiting the cells by liberating free anions and protons but also through the disordering of the membrane structure and thus possible membrane permeation effects (Piper *et al.*, 2001).

The very lipophilic nature of sorbic acid may be more inhibitory due to this increased ability to partition into the membrane structure and the accumulation of such compounds would undoubtedly affect the physico-chemical and consequently the function of the membrane. The accumulation of organic solvents has been shown to

affect the permeability of the membrane, but also disordering the membrane structure with possible deleterious effects on the conformation of key membrane bound enzymes - all having a negative effect on the cell (Weber & de Bont, 1996).

Another weak acid, benzoate has been suggested to form high anion concentrations and the release of free protons liberated from the dissociation of benzoic acid inhibited yeast growth by lowering the pH_i to such an extent that phosphofructokinase, and thus glycolysis, was inhibited (Krebs *et al.*, 1983).

Despite extensive study it is clear that the precise inhibitory action of the weak acid preservatives, in particular for sorbic acid on yeast growth is not yet clear, what does seem to be clear is that the inhibition is not solely due to one cellular process but a large number.

3.1.4. The effect of amphotericin B on pH_i

In contrast to sorbic acid, amphotericin B is not a weak acid, but a member of a group of compounds termed the polyene macrolide antibiotics, which are produced by members of the genus *Streptomyces*. Most of these compounds have antifungal activity but little antibacterial activity. They are characterised by large lactone rings (20-44 membered) combined with one sugar moiety (Bolard, 1986). It is the presence of polar hydroxyl groups and multiple hydrophobic double bonds that confer on the polyene antibiotics their characteristic features. Amphotericin B is a large, amphipathic molecule, which acts upon the membrane of target cells and importantly its efficacy is dependent upon interactions with membrane sterols. For instance, it is believed that yeasts are more sensitive to amphotericin B than to other polyenes because amphotericin B binds more avidly to the major sterol present in fungal membranes, ergosterol. The precise role of sterols in the antifungal activity of amphotericin B is still poorly understood (Bolard, 1986), but because bacterial cells have no sterols present in their membranes may explain why prokaryotes are resistant to this compound.

Various studies using membrane vesicles have indicated that amphotericin B forms a 'barrel' pore in the membrane (Hoogevest & Kruijff, 1978). This pore is made up of an assembly of molecules forming a cylindrical channel, with the hydrophilic side of the molecule facing into the channel, and the hydrophobic side interacting with the membrane lipids and the sugar moiety exposed to the external medium (a suggested structure for this 'barrel' pore is depicted in Appendix 1.0). The cylindrical channels are believed to consist of alternate sterol and antibiotic molecules (Bolard, 1986). A major consequence of the formation of these pores in the membrane is the dissipation of the proton gradient, loss of K^+ ions from the cell (Palacios & Serrano, 1978), and the leakage of additional cytoplasmic constituents, for example amino acids, Ca^{2+}, Mg^{2+} and $PO4_{3-}$ ions (Bolard, 1986). However, the effect of amphotericin B on the pH_i of growing yeast cells appears not to have been reported.

In addition to the development of the *in vivo* methodology to study pH_i, this Chapter also investigates the physiological effects and mode of action of different antifungal agents by comparing the inhibitory mode of action of two different antifungal compounds, sorbic acid (a weak-acid food preservative) and amphotericin B (a hydrophobic, membrane-active molecule). By determining whether the disruption of pH_i homeostasis in *S. cerevisiae* is a principal mechanism of growth inhibition, the understanding of the precise inhibitory action of these antifungal compounds from this work may ensure that novel and existing preservative molecules are selected that target the most vital aspects of cell physiology. In this way, the use of existing preservatives can be optimised and novel, synergistic combinations of preservatives can be designed that prevent yeast and mould spoilage that are a predominant issues in the beverage industry.

3.2. Results

3.2.1. pH_i calibration curves

To determine the pH_i of growing yeast cells, a calibration curve was first constructed of probe fluorescence against pH. Fluorescence intensity readings over an excitation spectrum between 400 nm and 500 nm at an emission wavelength of 525 nm were measured for the cleaved probe (carboxy fluorescein succinimidyl ester, CF-SE).

Figure 3.1 shows the pH-dependant excitation for this probe over a range of pH values in YNBG-CP media in the presence of permeabilised cells of *S. cerevisiae PMA1*. From this data, a ratio of the fluorescence intensity between the pH-dependent point (495 nm) and the pH-independent point (435 nm) was calculated and plotted as a function of pH to construct a ratio verses pH calibration curve. Ratio measurements have the advantage of eliminating a number of variables that lead to inaccuracies, including probe bleaching and variations in loading intensity between cells as well as variations in machine performance and biomass. The addition of permeabilised cells to reaction would hope simulate any possible quenching effects imposed by the cell presence in future experiments.

Figure 3.2 shows an average of three experimental calibration curves for the ratio values of the fluorescent intensity of CF-SE (495 / 435 nm) in YNBG-CP media with permeabilised cells of *S. cerevisiae PMA1* and plotted over a pH range of 7 to 4.5. The standard deviations for each of the calculated average ratio values and average calculated pH values are also shown. The experimental error for any given pH value calculated averaged to +/- 0.04 of pH unit and the highest error recorded was +/- 0.07 of pH unit.
Using the plotted data in Figure 3.2, a polynomial function using Microsoft Excel v. 5.2 was fitted to calculate intracellular pH (pH_i) (y) from the ratio values (x).
The equation best describing the calibration curve was:

$$y = 9E\text{-}05x^5 - 0.0038x^4 + 0.0592x^3 - 0.4304x^2 + 1.5803x + 3.5061$$

The regression (R^2 value) and indication of fit for this function was 0.9967.

3.2.2. Optimisation of loading *S. cerevisiae* with the fluorescent probe CFDA-SE

Samples of *PMA1* were loaded using a matrix of different conditions which included, CFDA-SE at 50 and 100 μM, incubated at 30, 35, 37 and 40°C for 0, 7, 12 and 24 hours in 100 mM citric / phosphate or CP buffer at pH 4.0 and 6.5, the levels of cellular fluorescence loaded into the samples were determined.

It was found that as the loading pH decreased from 6.5 to 4.0, the levels of intracellular fluorescence (loaded probe) increased (results not shown). Similarly, increasing the incubation temperature from 30 to 40°C, the CFDA-SE concentration from 50 to 100 μM and the duration of the incubation up to 24 hours, also significantly increased the level of intracellular fluorescence (results not shown).

The effect of different loading regimes on the cell viability is shown in Figure 3.3, and on subsequent cell growth in batch culture in Figure 3.4. Optimal fluorescent probe loading occurred after 24 hours at 40°C, pH 4.0 however, this regime did result in a minor loss of viability (Figure 3.3) compared to time 0 hours and also in a slight lag in the growth. But all of the regimes when compared had very similar specific growth rates (μ) (Figure 3.4 and see Table 3.1).

Table 3.1

Comparison of specific growth rates for cell growth after loading with different fluorescent probe loading regimes using spectrophotometer to measure growth

Probe Loading Regime	Specific Growth Rate (h^{-1})
Unloaded cells	0.48 h^{-1}
37°C / pH 6.5 CP Buffer / 50 μM CFDA-SE	0.47 h^{-1}
37°C / pH 4.0 CP Buffer / 50 μM CFDA-SE	0.52 h^{-1}
37°C / pH 6.5 CP Buffer / 100 μM CFDA-SE	0.49 h^{-1}
33°C / pH 6.5 CP Buffer / 100 μM CFDA-SE	0.47 h^{-1}
40°C / pH 6.5 CP Buffer / 100 μM CFDA-SE	0.53 h^{-1}

The loading conditions that were chosen for subsequent experiments were 100 μM CFDA-SE for 24 hours at 37°C, pH 4.0. These conditions were chosen as they gave maximal intracellular fluorescence without appreciably affecting cell viability, lag phase or subsequent growth rate.

3.2.3. Confirmation of fluorescent probe loading using CSLM

Visual analysis of the distribution and levels of intracellular fluorescence from the *PMA1* loaded cells is shown in Figure 3.5. Figure 3.5 a) shows a low magnification (480 x) phase-contrast image and a corresponding fluorescent image of a sample population of cells loaded with CFDA-SE by the above protocol. It is clear from the photograph the majority of the cells display high levels of intracellular fluorescence, which appears to be evenly loaded throughout the cytosol. This is confirmed by the pictures in Figure 3.5 b).

A higher magnification (1450 x) picture (Figure 3.5 b)) shows cell fluorescence super-imposed onto the corresponding phase image. As discussed above the intracellular fluorescence is evenly distributed across the cell, plus there is no evidence of probe compartmentalisation such as in the vacuole. A possible explanation for the presence of the apparently unstained cells could be that these cells have had their cell membranes disrupted, which with an external media pH of 4.0 would drastically reduce the internal fluorescence due to the pH equilibration across the membrane (see Figure 3.1). An alternative, though less likely reason, could be that a certain population of cells might have effluxed all of the probe back into the external environment.

3.2.4. Determination of pH_i

To test this newly developed *in vivo* pH_i method, cells of *PMA1* loaded with CFDA-SE probe were resuspended in 100 mM citric / phosphate buffer at pH 3.4 and incubated at 30°C for 30 min. The measured pH_i for these cells over the time period is shown in Figure 3.6. The initial pH_i was approximately 5.85, which compared favourably to measurements made by Cimprich *et al.* (1995), who used the fluorescent probe BCECF, and by Imai & Ohno (1995) using CFDA in buffer.

After 30 min of incubation at pH 3.4, 5.5 mM sorbic acid was added resulting in a rapid pH_i reduction of approximately 1 pH unit and the pH_i then remained constant for the duration of the experiment. Thus demonstrating the sensitivity of this method but also that this technique could produce results that were comparable to other similar referenced data.

The test was next repeated to determine the pH_i of cells with normal and reduced H^+-ATPase expression (strains *PMA1* and *pma1-205* respectively) after resuspension in 100 mM citric / phosphate buffer at a higher pH of 4.5 for 30 min (Figure 3.7). The initial pH_i in *PMA1* was approximately 6.1, in *pma1-205* was approximately 5.9 (0.2 units lower). In both strains 5.5 mM sorbic acid addition resulted in a significant and instantaneous drop in pH_i of approximately 0.65 pH unit.

In the third experiment, pH_i and $O.D._{600}$ were simultaneously measured for growing cells of *PMA1* and *pma1-205* in YNBG-CP, pH 3.8 at 30°C, this time without the addition of any sorbic acid. Figure 3.8 depicts a typical plot of logarithmic growth and intracellular pH on separate axis against time. After an initial lag phase of approximately 2 hours, *PMA1* entered exponential growth. Simultaneous determination of pH_i showed that this initially stabilised at just under pH 6.0, but declined gradually during exponential growth to approximately pH 5.6. In comparison, *pma1-205* had an extended lag period of approximately 25 hours before entering exponential growth. During this lag period the initial pH_i was approximately 5.4. This pH_i of *pma1-205* declined further to approximately pH 5.0 immediately prior to the onset of exponential growth and then declined gradually during exponential growth to approximately pH 5.0. Another such plot as a further example is shown in Appendix 2.1, although this time the experiment was performed at a media pH of 4.5. Again a similar pattern of growth rates and intracellular pH can be seen, although the pH values are the stabilised intracellular pH's are approximately 0.1 pH unit higher.

Note: All specific growth rate measurements in this Chapter were deduced by using a spectrophotometer to measure optical density at 600nm (section 2.3.2).

3.2.5. Effect of antifungal compounds on the pH_i of yeast cells

3.2.5.1. The effect of exposure to increasing concentrations of sorbic acid and amphotericin B on the growth rate and pH_i of S. cerevisiae

Increasing concentrations of sorbic acid (0 to 1.6 mM undissociated acid) and amphotericin B (0 to 9.5 μM) were added to growing cultures of *S. cerevisiae PMA1* in YNBG-CP medium at pH 4.5 and 3.8. An example of the raw data obtained for two concentrations of amphotericin B compared to a control at a culture pH of 3.8 is shown in Figure 3.9. The graph depicts logarithmic growth of *PMA1* and change in pH_i as a result of addition of the inhibitor both plotted against time. The initial starting pH_i was approximately 5.7. Similar experiments were carried out with sorbic acid at pH 3.8 and with sorbic acid and amphotericin B at a culture pH 4.5 (these results are shown in the Appendices as examples, Appendices 2.2, 2.3 and 2.4). Again similar initial pH_i and patterns of growth and data changes were observed. Appendix 2.4 shows two experiments over laid together and the scatter of data points around an average line indicating general experimental variation in this data.

From the data generated in these experiments, post-additive growth rates and the change in pH_i due to the addition of each compound were calculated, as shown in Figure 3.10 a) and b). At both pH 4.5 and 3.8, the effect of increasing sorbic acid addition resulted in significant inhibition of growth (Figure 3.10 a)). However, by expressing each sorbic acid concentration as the concentration of acid undissociated at pH 4.5 and 3.8, it is clear from Figure 3.10 a) that the growth inhibition is equal at both pH values and is therefore dependent on the concentration of the undissociated sorbic acid present. Simultaneous determination of change in pH_i revealed only small reductions at both pH 4.5 and 3.8, in particular for the concentrations of undissociated sorbic acid that gave the greatest inhibition of growth (Figure 3.10 a)).

As with sorbic acid addition, exposure to increasing concentrations of amphotericin B (in particular up to 1 μM) caused considerable growth inhibition of at both pH's (Figure 3.10 b)). The degree of growth inhibition was similar regardless of the culture pH. However, in contrast to sorbic acid, the same concentration of amphotericin B reduced pH_i much more at pH 3.8 as compared to 4.5.

3.2.5.2. The effect of increasing undissociated sorbic acid or amphotericin B at fixed pH values on the growth rate and pH_i of S. cerevisiae

The inhibitory effects of exposure of the yeast to increasing concentrations of sorbic acid and amphotericin B were compared. Sorbic acid was added to the *PMA1* cultures to concentrations up to 1.0 mM and amphotericin B to final total concentrations up to 9.5 µM, in the final solution. The post-additive growth rates of *PMA1* grown in YNBG-CP, at culture pH values of 4.5 and 3.8, are plotted against the actual pH_i values measured at the end of each experiment for each concentration of the two compounds (Figure 3.11).

At pH 4.5 and 3.8, increasing concentrations of sorbic acid significantly reduced the growth rate (*x*-axis) compared to a control culture, but had little effect on pH_i (*y*-axis). Thus, there is no correlation between the degree of growth inhibition induced by sorbic acid and the change in pH_i (Figure 3.11). In contrast, increasing concentrations of amphotericin B reduced growth rates and there was a correlation between the reduction in growth rate and the decline in pH_i, particularly at the lower culture pH of 3.8 (Figure 3.11).

3.2.5.3. The effects of the same concentration of undissociated sorbic acid or amphotericin B on growth rate and pH_i as a function of culture pH

The effects of changing culture pH (5.0, 4.5, 4.2, 3.8 and 3.2) on the inhibitory effects of 0.22 mM undissociated sorbic acid and 0.35 µM amphotericin B on *PMA1* grown in YNBG-CP were compared. 0.22 mM undissociated sorbic acid and 0.35 µM amphotericin B were selected because these concentrations inhibited growth and pH_i effectively at pH 4.5 and 3.8 in the previous experiments. Results are shown in Figures 3.12 a) and b). As in Figure 3.11, the specific growth rates for the different culture pH values of 5.0 to 3.2 as a result of the inhibitor were plotted against actual pH_i values measured at the end of each experiment.

The control cultures showed that decreasing the culture pH from 5.0 to 3.8 had an increasing effect on the specific growth rates, however, this was more noticeable for pH 3.2 (points moving from right to left). In contrast, decreasing culture pH had no

observable marked effect on pH_i, even at pH 3.2 where growth rate was more reduced (Figure 3.12 a) and b)). These results are in agreement with previous referenced work that shows only minor changes in pH_i result from decreasing the culture pH (Malpartida & Serrano, 1981 and Imai & Ohno, 1995) but not with Cimprich *et al.,* 1995.

As shown in Figure 3.12 a), 0.22 mM undissociated sorbic acid inhibited growth at all culture pH's tested (line shifted across the *x* axis in comparison to the control). However, this inhibition of growth was dependent on decreasing culture pH. For example, in the presence of 0.22 mM undissociated sorbic acid, the growth rate at pH 3.2 was reduced by approximately 50% as compared to that at pH 5.0 (Figure 3.12 a)). Significantly, this inhibition of growth did not appear to be due to the change in pH_i, as this remained virtually unchanged across the culture pH values tested, although at pH 3.2 an increased dip in pH_i was noted.

0.35 μM amphotericin B also inhibited growth rate in a manner dependent on decreasing culture pH. In the presence of 0.35 μM amphotericin B, growth rate at pH 3.2 was reduced by approximately 70% as compared to that at pH 5.0 (Figure 3.12 b)). In contrast to 0.22 mM sorbic acid, 0.35 μM amphotericin B caused reduction in pH_i at all culture pH values tested and the magnitude of this reduction in pH_i was dependent on the culture pH. Therefore, unlike with sorbic acid, there is some correlation between the inhibition of growth rate and reduction in pH_i caused by amphotericin B.

3.2.6. Effect of prior exposure to sorbic acid or amphotericin B in the yeast membrane permeability at low pH

Many studies have proposed that the inhibitory action of amphotericin B is due to disruption of the cytoplasmic membrane. Therefore, to determine if membrane disruption was contributing to the inhibitory action of amphotericin B and sorbic acid at the concentrations used in this study, measurements were taken of the net proton flux of cell suspensions exposed to these compounds at different pH values of 5.0, 4.2 and 3.2. These results are shown in Figure 3.13.

At pH 5.0 there was little net influx or efflux of protons across the cell membrane under any conditions. Similarly, at pH 4.2 untreated cells showed little net proton flux. However, prior exposure to sorbic acid and amphotericin B at this pH resulted in a minor increase in the net proton influx (or membrane permeability).
As expected, at an external pH of 3.2, the net proton influx in the untreated cells was increased compared to that at 4.2, i.e. due to the increased proton concentration externally. Exposure to sorbic acid and amphotericin B at pH 3.2 also resulted in a significant increase in net proton influx compared to the same treatment at pH 4.2.

3.2.7. Effect of exposure of sorbic acid and amphotericin B on the intracellular ADP / ATP ratio levels in yeast cells

Studies have suggested that observed reductions in the cellular ATP pool upon exposure to sorbic acid could be due to the activation of the membrane H^+-ATPase (Holyoak *et al.,* 1996 and Piper *et al.,* 1997). Furthermore, it has been proposed that the depletion of cellular ATP in yeast cells occurring as a consequence of the activation of this enzyme by stress factors that disrupt pH_i homeostasis could result in growth inhibition (Piper *et al.,* 1997).

To study this possibility, the effect of addition of sorbic acid and amphotericin B on the intracellular ADP / ATP ratio of *PMA1* was measured (Figure 3.14).
The untreated control culture growing in YNBG-CP at pH 3.2 had an ADP / ATP ratio of approximately 0.9 and this declined gradually during exponential growth to a value of approximately 0.6. The addition of 0.22 mM undissociated sorbic acid to *PMA1* resulted in a decrease in growth rate (as observed previously) and a rapid increase in the ADP / ATP ratio to a maximum value of approximately 1.6 (thus indicating an increased use of ATP). After 3 hours the ADP / ATP ratio declined to the levels observed in the untreated control culture.

The addition of 0.35 µM amphotericin B resulted in an increase in the ADP / ATP ratio. However, unlike sorbic acid there was a delay of approximately 30 min before any increase in the ratio was observed and appeared to stay relatively high for the duration of the experiment. As with sorbic acid, observable inhibition of growth rate appeared to correlate with the increase in the ADP / ATP (Figure 3.14).

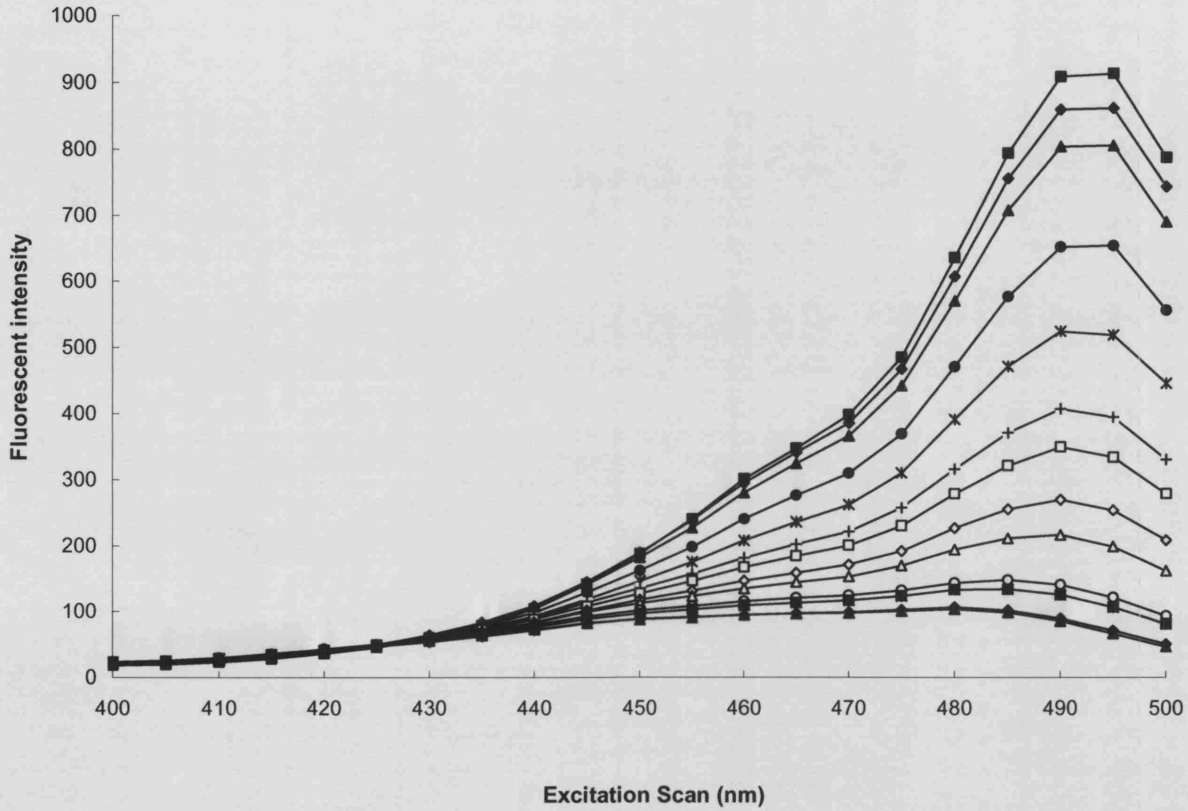


Figure 3.1

The pH-dependant excitation spectra of CF-SE in YNBG-CP in the presence of permeabilised cells of *S. cerevisiae PMA1*. The pH values were; 7.00 (■), 6.75 (♦), 6.64 (▲), 6.40 (●), 6.21 (✶), 6.00 (+), 5.88 (□), 5.71 (◊), 5.54 (Δ), 5.23 (o), 5.14 (×), 4.80 (■), 4.76 (♦), 4.57 (▲).

The emission wavelength was set at 525 nm.

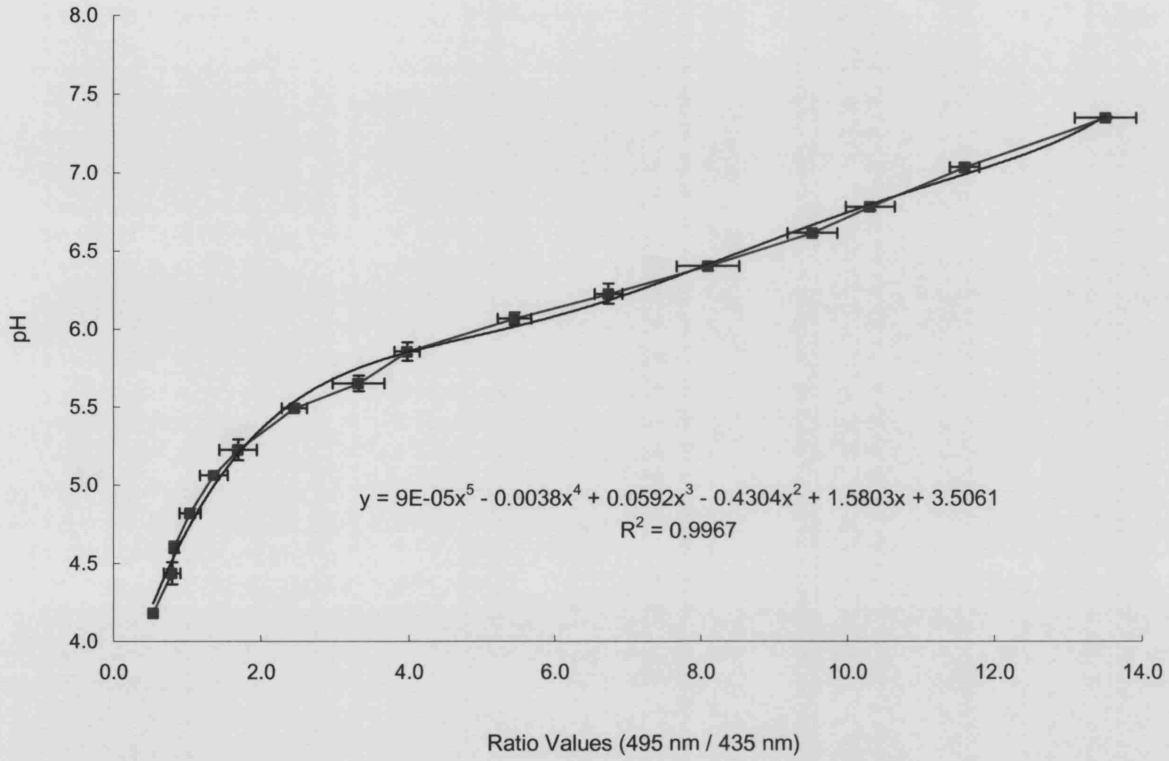


Figure 3.2

Calibration curve of the fluorescent intensity ratio of CF-SE (495 / 435 nm) vs. pH (■) in YNBG-CP with permeabilised cells of *S. cerevisiae PMA1*. (-). Graph showing results for triplicate experiments with standard error bars for both the calculated ratio value and pH. The fitted equation best describing the calibration curve is also shown.

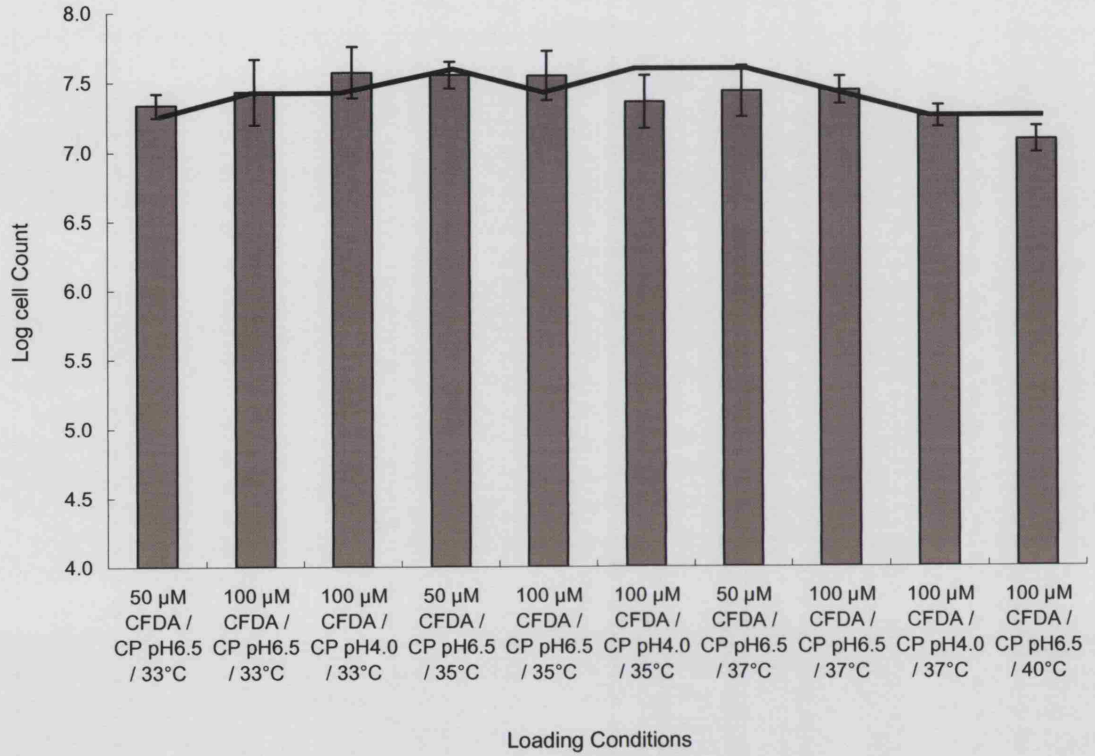


Figure 3.3

The effect of CFDA-SE loading conditions on the viability of *S. cerevisiae PMA1* after 24 hours incubation under the loading conditions noted on the graph. The conditions used were either 50 µM to 100 µM CFDA-SE in 100 mM citric / phosphate buffer, at either pH 6.5 or pH 4.0 and at temperatures ranging from 33°C to 40°C. Graph depicts the average counts for three experiments and line for the 0 hrs start counts.

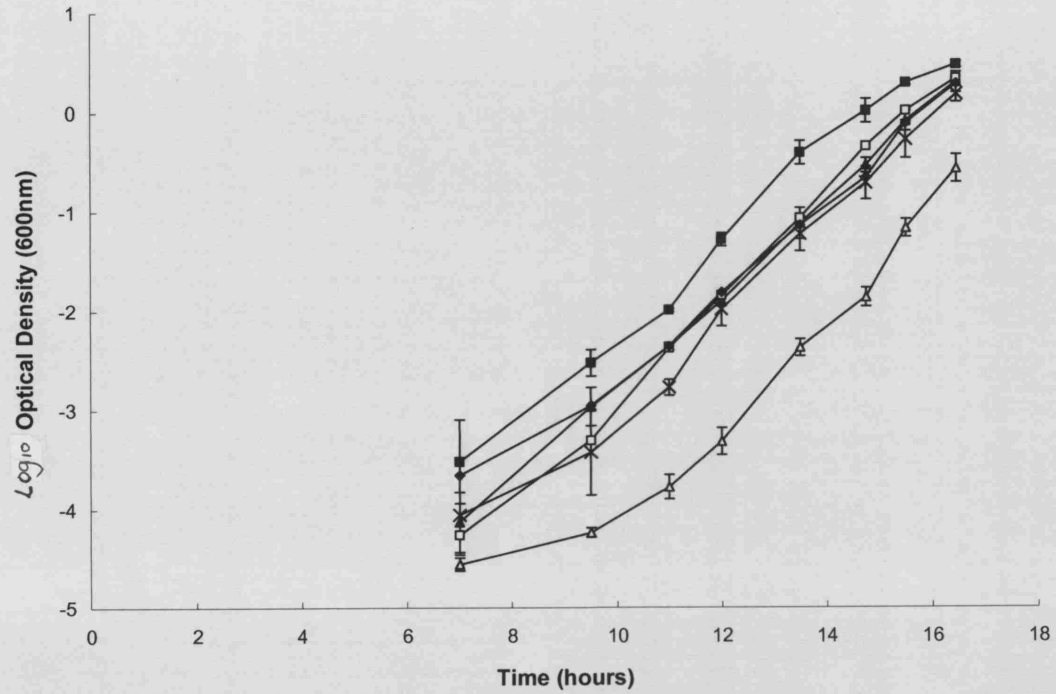


Figure 3.4

The effect of CFDA-SE loading conditions on the subsequent outgrowth of *S. cerevisiae PMA1* in YEPD (inoculum size 5.0 x 10^4 cells ml^{-1}), pH 5.7 after 24 hour incubation with the conditions; 100 µM CFDA-SE in 100 mM CP buffer, pH 6.5 at 33°C (♦), 50 µM CFDA-SE in 100 mM CP buffer, pH 6.5 at 37°C (▲), 100 µM CFDA-SE in 100 mM CP buffer, pH 6.5 at 37°C (●), 100 µM CFDA-SE in 100 mM CP buffer, pH 4.0 at 37°C (□) and 100 µM CFDA-SE in 100 mM CP buffer, pH 6.5 at 40°C (△), compared to an untreated control culture (■).

Graph depicts the average counts for three experiments with standard error bars.

a)

b)

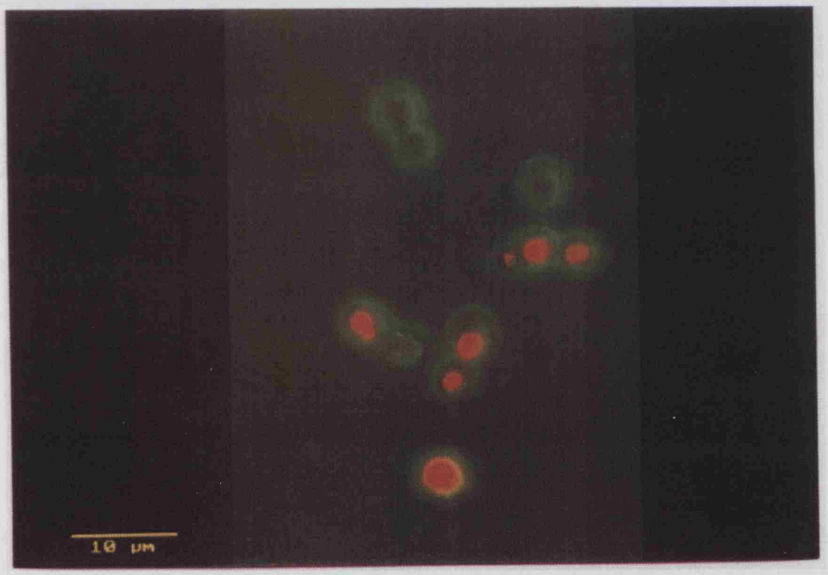


Figure 3.5 a) and b)

Images of *S. cerevisiae PMA1* cells after loading with CFDA-SE (100 µM in 100 mM CP buffer, pH 4.0, at 37˚C for 24 hours) taken using confocal scanning laser microscopy (CSLM) showing:

a) a simultaneous phase contrast image (left) and a corresponding fluorescent image (right). (Scale bar representing 50 µm).

b) a fluorescent colour image super-imposed onto a simultaneous phase image. (Scale bar representing 10 µm).

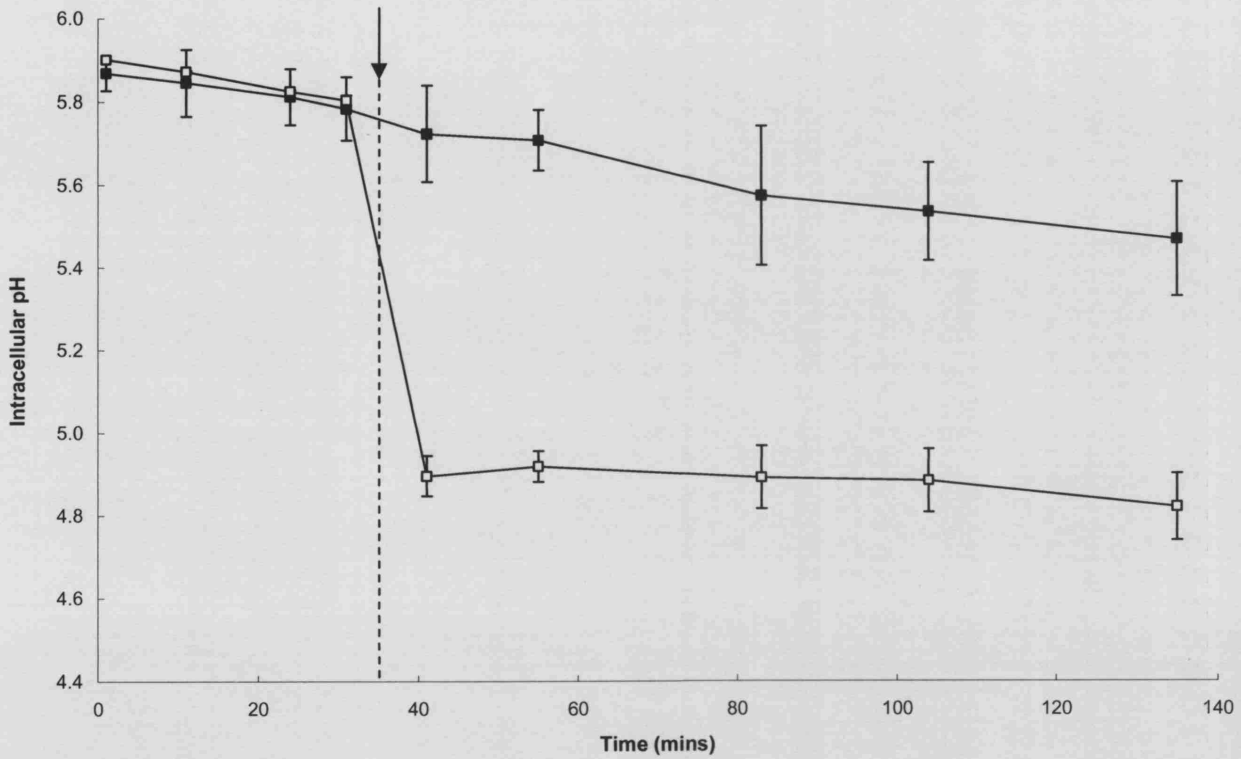


Figure 3.6

A plot showing the intracellular pH of *S. cerevisiae PMA1* in 100 mM citric / phosphate buffer, pH 3.4 at 30°C (■), and after addition of sorbic acid (5.5 mM) (□). The dotted line and arrow indicate the time of addition for the sorbic acid (approx. 30 min). The data representing triplicate experiments with standard error bars on the graph indicating the variation of this data.

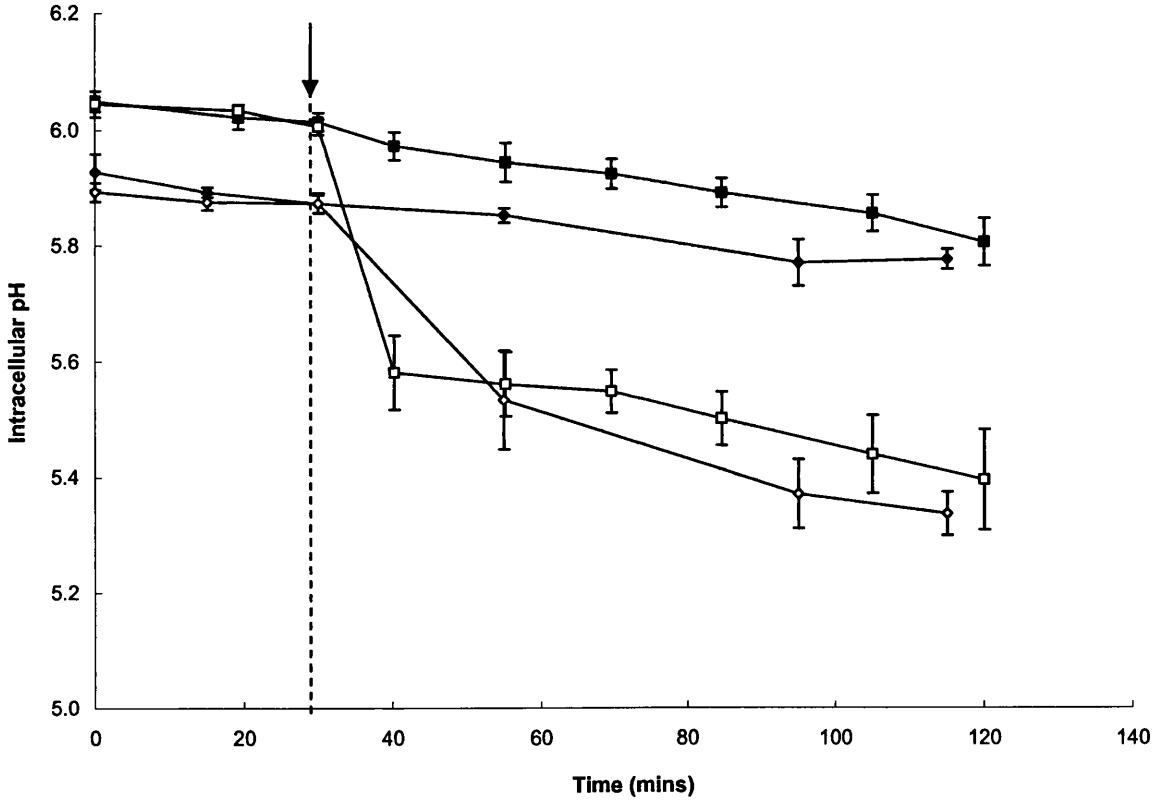


Figure 3.7

Intracellular pH of *S. cerevisiae PMA1* (■, □) and *pma1-205* (♦, ◊) in 100 mM citric / phosphate buffer, pH 4.5 at 30°C (solid symbols) and after addition of sorbic acid (5.5 mM) (open symbols). The dotted line and arrow indicate the time of addition of sorbic acid (approx. 30 min). The data representing triplicate experiments with standard error bars on the graph indicating the variation of this data.

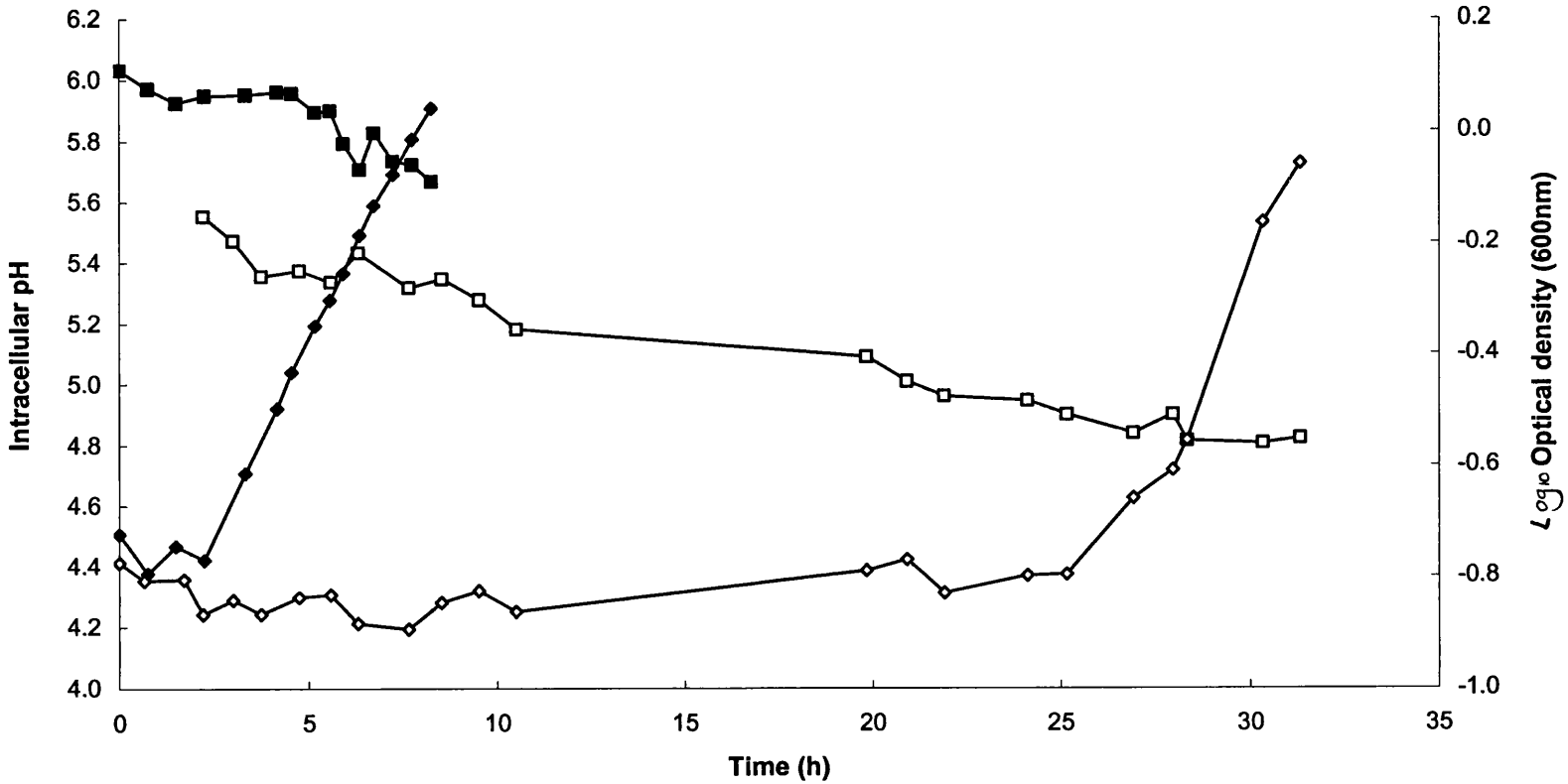


Figure 3.8

Simultaneous measurement of logarithmic growth (ln optical density 600nm) (♦, ◊) and intracellular pH (■, □) of *S. cerevisiae PMA1* (solid symbols) and *pma1-205* (open symbols) in YNBG-CP, pH 3.8 at 30°C. This graph is representative of a typical plot.

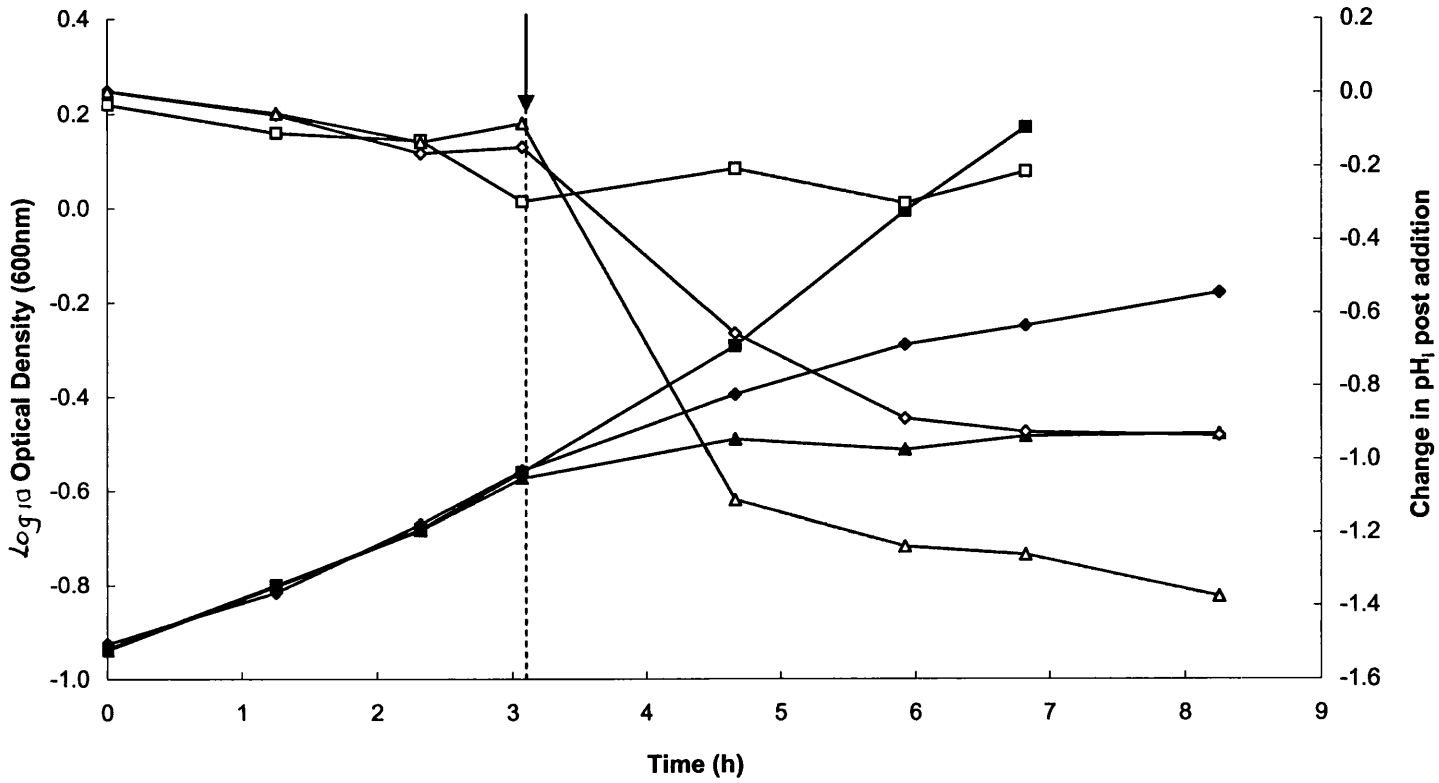


Figure 3.9

The effect of addition of 0 (■,□), 0.712 µM (♦, ◊) and 4.5 µM (▲, Δ) amphotericin B on the growth (solid symbols) and change in pH_i post addition (open symbols) of *S. cerevisiae PMA1* in YNBG-CP at pH 3.8. The dotted line and arrow indicates the point of addition of amphotericin B. The initial starting pH_i was approximately 5.7. The graph represents a typical plot.

a)

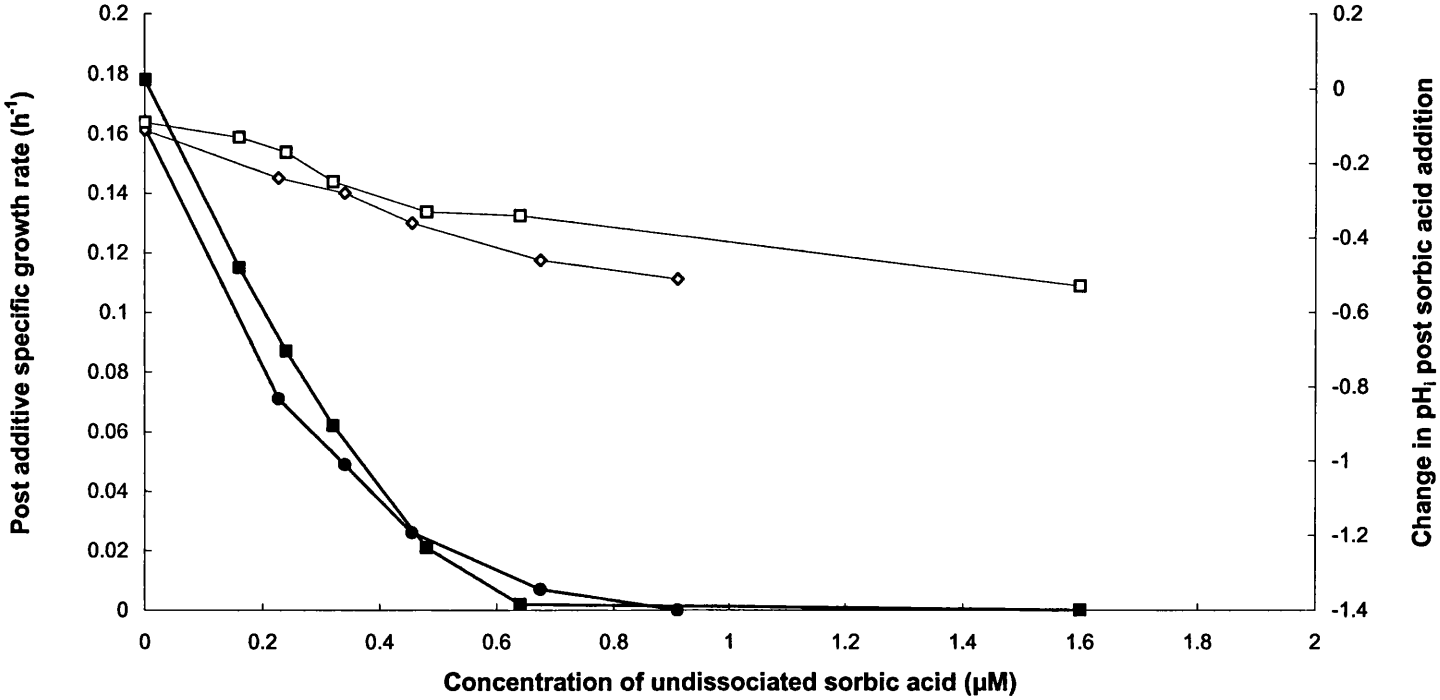


Figure 3.10 a)

The effect of increasing concentrations of undissociated sorbic acid on post additive specific growth rate (solid symbols) and change in pH_i post sorbic acid addition (open symbols) of *S. cerevisiae PMA1* in YNBG-CP at pH 4.5 (■,□) and 3.8 (♦, ◊). This graph represents typical data for both pH values.

b)

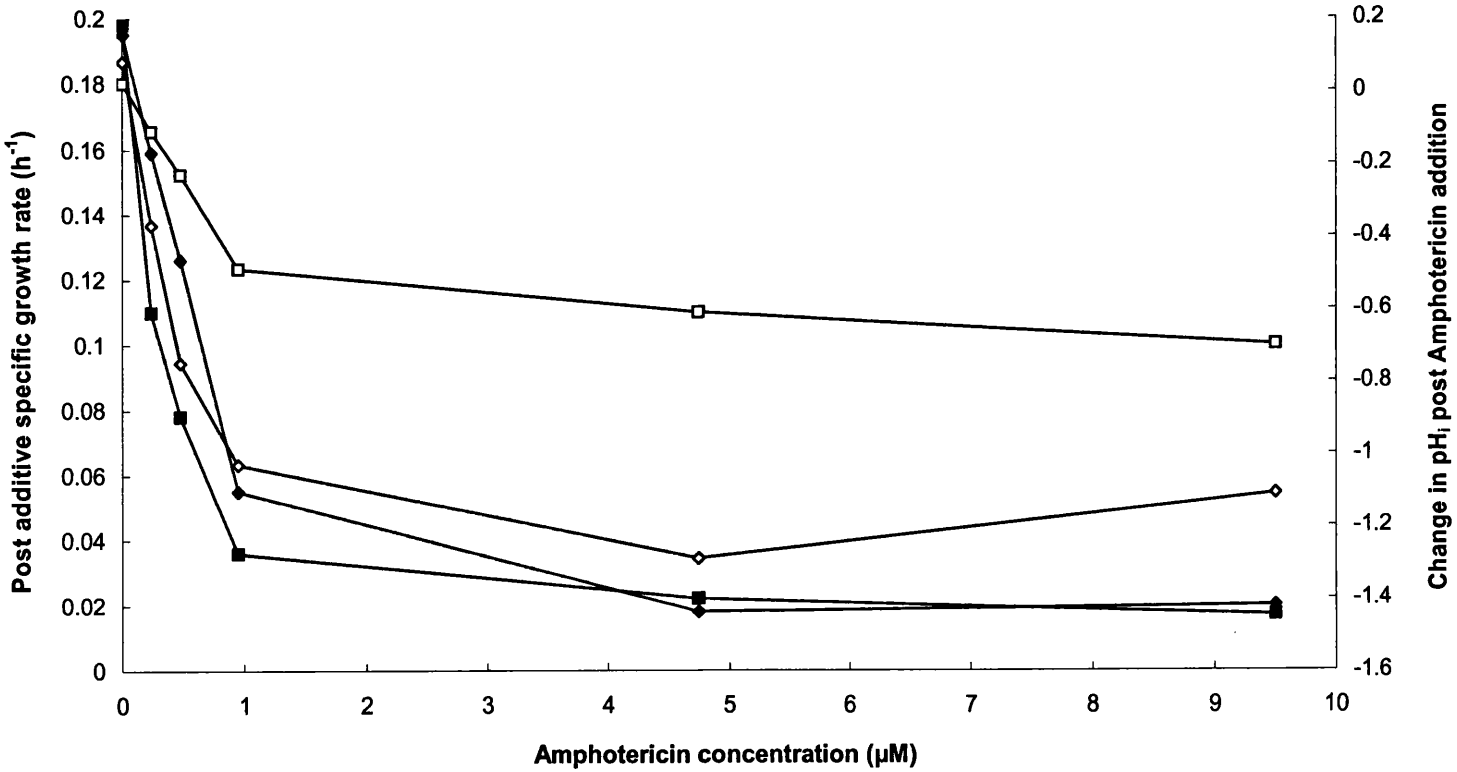


Figure 3.10 b)

The effect of increasing concentrations of amphotericin B on post additive specific growth rate (solid symbols) and change in pH_i due to amphotericin addition (open symbols) of *S. cerevisiae PMA1* in YNBG-CP at pH 4.5 (■,□) and 3.8 (♦, ◊). This graph represents typical data for both pH values.

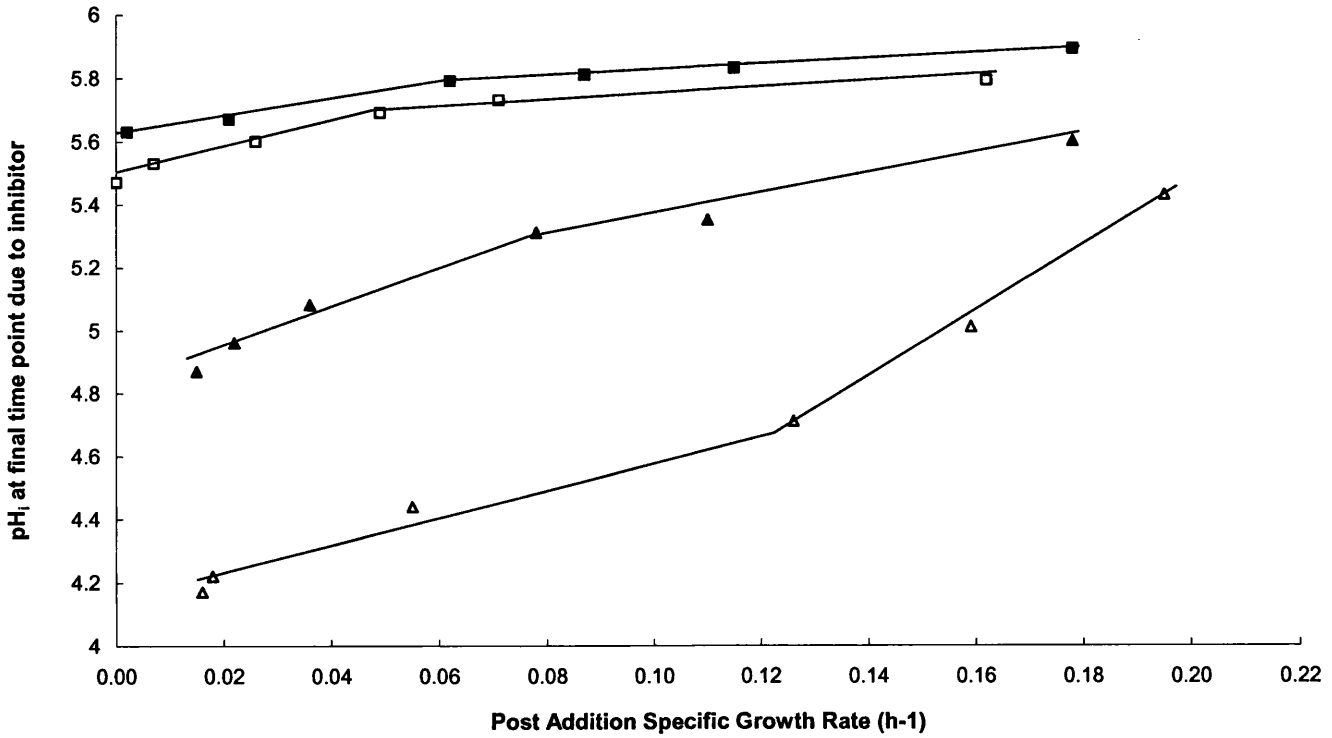
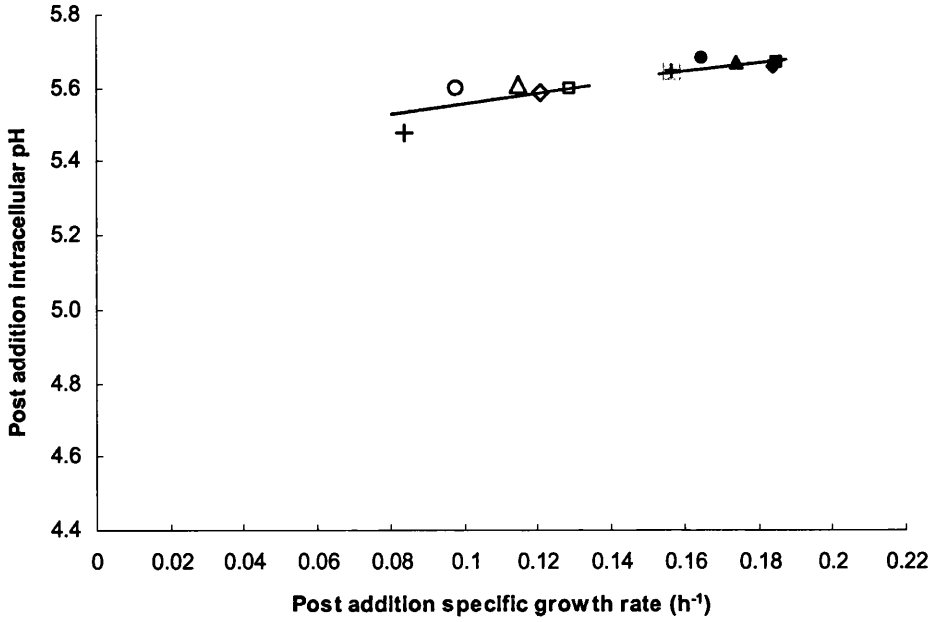


Figure 3.11

The relationship between specific growth rate and final pH_i value of *PMA1* after addition of increasing concentrations of sorbic acid (squares) and amphotericin B (triangles) at pH 4.5 (■, ▲) and pH 3.8 (□, Δ). Sorbic acid was added to final total concentrations of 0, 0.12, 0.25, 0.37, 0.5, 0.75 and 1.0 mM. Amphotericin B was added to final total concentrations of 0, 0.23, 0.43, 0.71, 0.95, 4.5 and 9.5 µM. The effect of increasing concentrations of each compound is plotted sequentially from the right side of the graph. This data is representative of triplicate experiments.

a)



b)

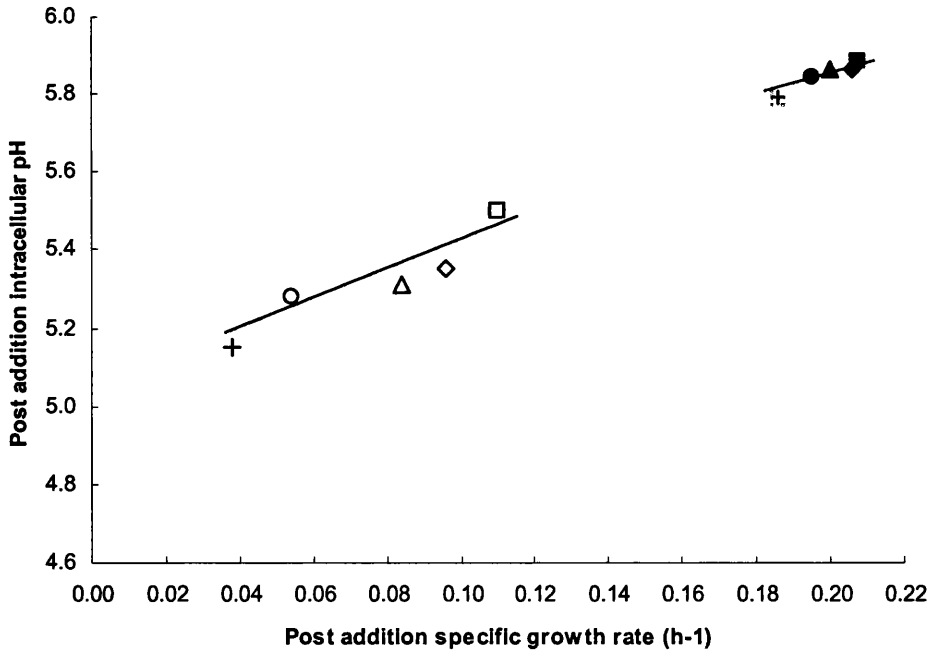


Figure 3.12 a) and b)

Relationship between specific growth rate and pH_i of *S. cerevisiae PMA1* after addition of a) 0.22 mM undissociated sorbic acid, and b) 0.35 µM amphotericin B, at pH 5.0 (□), 4.5(Δ), 4.2 (◊), 3.8 (○) and 3.2 (+). In each case, this was compared to the effect of culture pH alone at pH 5.0 (■), 4.5 (▲), 4.2 (♦), 3.8 (●) and 3.2 (±) on the relationship between growth rate and pH_i. These graphs represent typical plots.

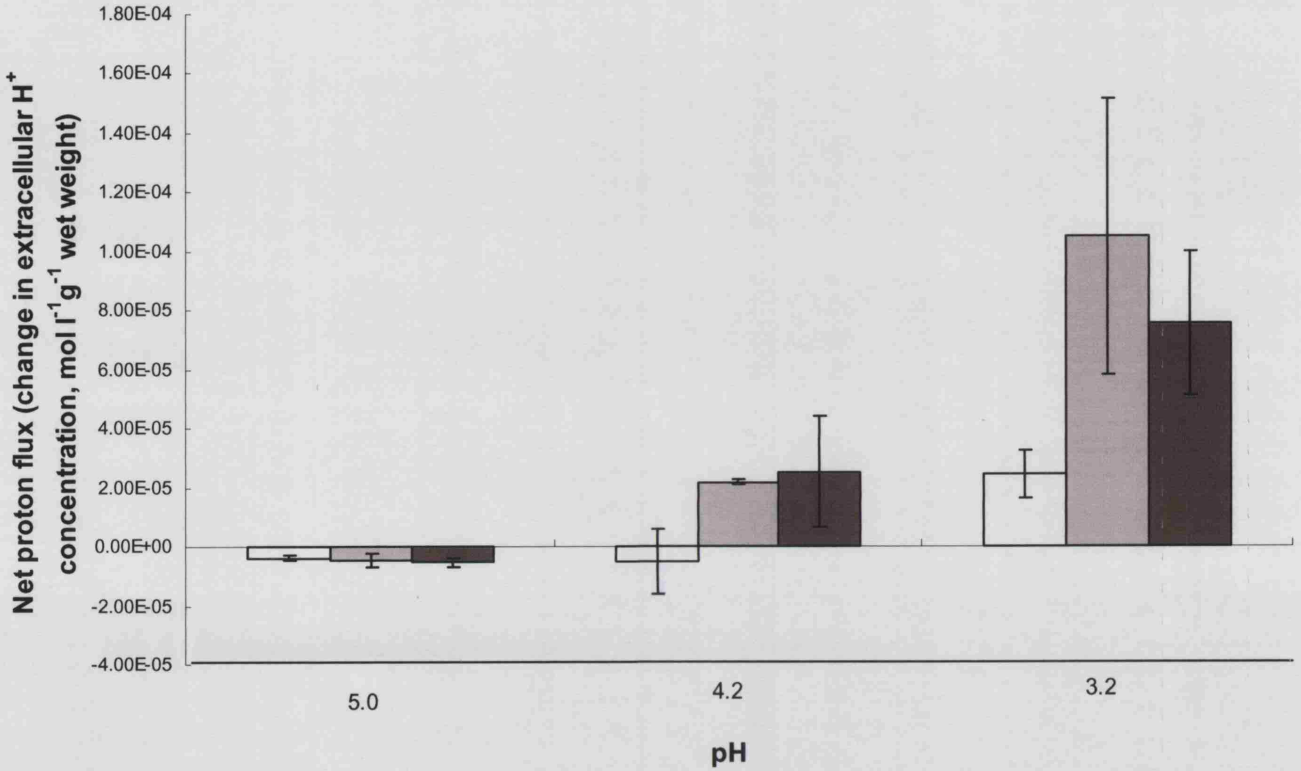


Figure 3.13

The effect on the membrane permeability of *S. cerevisiae PMA1* after pre-incubation with 0.22 mM undissociated sorbic acid (grey bars) and 0.35 µM amphotericin B (black bars) measured by net proton flux at pH 5.0, 4.2 and 3.2 compared to an untreated control (white bars). A negative value is indicative of net proton efflux from the cells and a positive value, net proton influx. The graph represents triplicate data with standard error bars on the graph indicating the variation of this data.

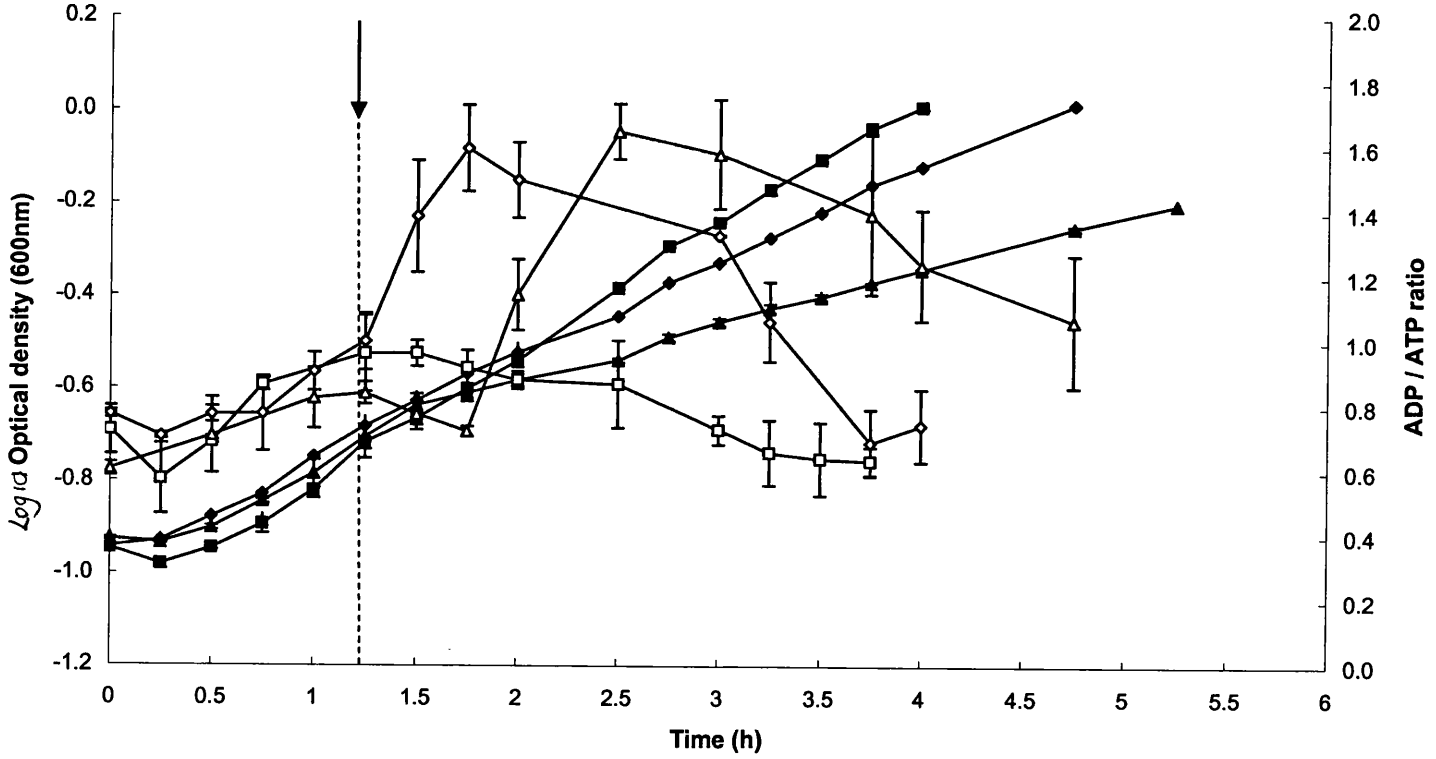


Figure 3.14

The effect on the growth (solid symbols) and ADP / ATP ratio (open symbols) of *S. cerevisiae PMA1* growing in YNBG-CP at pH 3.2 after the addition of 0.22 mM undissociated sorbic acid (◆, ◇) and 0.35 µM amphotericin B (▲, Δ), compared with an untreated control (■, □). The dotted line and arrow indicates the point of addition of sorbic acid and amphotericin B. Graph representing triplicate sample results with standard error bars on the graph indicating the variation of this data.

3.3. Discussion

3.3.1. Development of an *in vivo* fluorescent intracellular pH detection method

This Chapter firstly describes the successful adaptation of a fluorescent intracellular pH (pH_i) technique first developed by Breeuwer *et al.* (1996) in bacteria to the application in the spoilage yeast *Saccharomyces cerevisiae*. Unlike previous methods such as the distribution of radio-labelled weak acids and ^{31}P-NMR, this technique allows for the rapid and simple assay of the effect of antifungal compounds on the pH_i in growing cells, thus representing a totally non-invasive and *in vivo* methodology.

This technique is based upon the pH-dependent intracellular fluorescence of a novel fluorescent dye, CF-SE, which is loaded into the cytosol of the cell. The principal advantage of CF-SE over other probes, such as CF and BCECF, is that the succinimidyl group (SE) of the probe is believed to form conjugates with the aliphatic amines on proteins and other intracellular molecules in the cell (Haughland, 1996). The use of such a conjugated probe allows for the measurement of pH_i under conditions that might lead to damage to cell membranes and would otherwise lead to the complete leakage of the probe from the cells to the external environment (Breeuwer *et al.*, 1996).

Extensive studies of probe loading conditions revealed that maximum levels of intracellular fluorescence were achieved at the highest concentration of CFDA-SE tested (100 μM). This supports the observations made by Breeuwer *et al.* (1995), who found that CFDA probe uptake into the cell, most likely occurred via passive diffusion. Breeuwer *et al.* (1996) were able to load CFDA-SE into bacterial cells simply by incubating cells in HEPES buffer, pH 8.0 for 10 min at 30°C. However, under similar conditions with *S. cerevisiae* it was impossible to load similar amounts of CFDA-SE into the cells, perhaps due to the more complex nature of the yeast cell wall (results not shown). Optimal loading of probe only occurred if the cells were incubated at heat shock temperatures, e.g. 37 and 40°C, for 24 hours in 100 mM citric / phosphate buffer, pH 4.0. It is well documented that stress regimes such as

heat shock results in increased membrane and cell wall disruption in yeast and moulds leading to enhanced uptake of molecules that are otherwise largely excluded, for example, ethidium bromide (Coote *et al.*, 1994) and propidium iodide (Brul *et al.*, 1997). Thus it was necessary to select a probe loading regime of 37°C for 24 hours at pH 4.0 that did not significantly effect either cell viability (Figure 3.3) or subsequent outgrowth in batch culture (Figure 3.4) under optimal conditions. Furthermore, it was important to allow the yeast cells to recover and stabilise following this imposed stress of the probe loading conditions, prior to all experiments loaded cells were incubated for 30 min at 30°C.

A recurring problem with many techniques is the efflux of the probe from the cytosol of the loaded cell back into the external medium, resulting in high background fluorescence which, if ignored, can result in inaccurate determination of pH_i.
In *S. cerevisiae*, the efflux of fluorescent probes out of the cell is believed to be catalysed by an energy dependent, carrier-mediated, transport mechanism in the yeast cell (Breeuwer *et al.*, 1994). This mechanism is also thought to be the basis of resistance to a broad spectrum of functionally and structurally unrelated drugs in yeast. All of these effects are mediated by a network of ATP-dependent membrane proteins termed the pleiotrophic drug resistance (PDR) network (Piper *et al.,* 1998) and are able to extrude toxic compounds from the cell (Balzi & Goffeau, 1995), including the fluorescent probes. Thus the use of a conjugated probe in this instance over came some of the issues from probe efflux from the cell. It was clear from the photographs in Figure 3.5 that the probe was definitely located in the cytosol and very little was located externally.

However, after a number of hours performing such experiments, a low level of probe was effluxed from the cell. Thus to overcome this difficulty, the pH_i determination of cells was carried out in citric / phosphate buffer or in buffered media (YNBG-CP), over a range of pH values from 4.3 to 3.2. At these external pH values, any cleaved fluorescent probe (CF-SE) that was effluxed from the cells into the external media had little resultant fluorescence and thus erroneous measurements due to background fluorescence were not a significant problem (see Figure 3.1). However, at pH 4.5 some background fluorescence was detected in cells growing in YNBG-CP. Thus to eliminate this problem, for each sample, a total fluorescence value was recorded

followed by a fluorescence reading of the culture supernatant after removal of the cells by centrifugation. Any background fluorescence detected was then subtracted from the total fluorescence reading and eliminated from subsequent calculations of pH_i.

All of the experiments performed to test this methodology showed that the probe was successfully loaded and was not immediately effluxed out of the cell. Any issues with probe leakage and thus erroneous readings could be removed either by using low external pH media or subtracting the background fluorescence values from any data.

This technique did however have a couple of drawbacks, this being amount of samples that could be measured during an experiment and thus the number of replicates that could be performed. Due to this issue, many of the experiments depicted were performed over a number of days.

3.3.2. Comparison of the newly developed *in vivo* fluorescent pH_i method to results produced by other studies

To determine whether the resultant pH_i values measured were reliable using such a technique for yeast cells, the results (Figure 3.6 in buffer and 3.8 in YNBG media) were compared to other studies in the literature. This comparison revealed that the values did compare favourably with a number of different but comparable studies carried out using a fluorescence microscope image processing technique. For example, Imai & Ohno (1995), measured readings of approximately 5.8 in a complex salts buffer at pH 4.5 and Cimprich *et al.* (1995) observed a pH_i value of approximately 5.3 in 0.2 M triethanolamine-phthalate buffer at pH 4.3. Confirming the studies of Cimprich *et al.* (1995), the pH_i values reported in this study were approximately 1 pH unit lower than those measured by ^{31}P-NMR spectroscopy (Gillies *et al.*, 1981 and Coote *et al.*, 1994). A possible explanation for these differences could be that the high cell densities employed in these NMR studies resulted in the cells being in a non-physiological state thus affecting the pH_i values. Thus giving confidence to the resultant pH_i values calculated both by the developed technique. In this Chapter the technique was then applied to investigate the effect of different antimicrobial on the pH_i of the yeast cell.

3.3.3. The effect of sorbate on the pH_i of static and growing yeast

The weak-acid food preservative, sorbic acid is known to reduce the pH_i of yeast cells (Krebs *et al.*, 1983, Cole & Keenan, 1987 and Booth & Kroll, 1989). Upon the addition of sorbic acid to CF-SE loaded cells suspended in biological buffer, large instantaneous reductions in pH_i were detected, thus confirming the effectiveness of this pH_i methodology to be able to rapidly and non-invasively measure pH_i fluctuations in *S. cerevisiae* (Figures 3.6 and 3.7).

As a further validation of this methodology, the pH_i was measured in a reduced-expression membrane H^+-ATPase mutant (*pma1-205*) and compared to the values observed with the isogenic parent, *PMA1*. In yeast, the plasma-membrane H^+-ATPase plays a major role in the maintenance of pH_i homeostasis by coupling ATP hydrolysis to the expulsion of protons, generating a proton gradient (Serrano, 1984). Unsurprisingly, the enzyme has been shown to be critically involved in resistance to stress factors that disturb pH_i homeostasis, for example heat (Coote *et al.*, 1994), sorbic acid (Holyoak *et al.*, 1996) and octanoic acid (Viegas & Sa-Correia, 1991).

The comparison of the pH_i of *PMA1* and *pma1-205,* which were incubated in citric / phosphate buffer at pH 4.5, revealed that the mutant strain had a significantly reduced pH_i (Figure 3.7). The *pma1-205* mutant has been shown to have 66% less membrane H^+-ATPase activity compared to the parent (Vallejo & Serrano, 1989) possibly explaining this effect. This result also confirmed the studies of Vallejo & Serrano (1989) who determined pH_i by the distribution of radio-labelled weak acids. The addition of 5.5 mM sorbic acid resulted in a large drop in pH_i, in both strains, to a similar value of approximately 5.3. The drop in pH_i was less in the reduced-expression H^+-ATPase mutant possibly because the initial pH_i value was reduced compared to the parent strain. According to weak acid theory and explaining these results, the same concentration of undissociated sorbic acid would enter both strains but would dissociate to a lesser extent in the mutant strain, due to the lower pH_i, thus resulting in a smaller overall drop in pH_i.

Following experiments in buffer, pH_i was successfully measured in growing cells of *PMA1* and *pma1-205* (Figure 3.8). As shown previously (Holyoak *et al.*, 1996), the reduced-expression of the membrane H^+-ATPase resulted in significant extension of

the lag phase compared to the wild-type parent. Measurement of pH_i during the lag and growth phases of both strains revealed a lower pH_i in the membrane H^+-ATPase mutant, thus confirming the results observed in buffer. However, the reduced pH_i in the mutant does not appear to account for the extended lag phase in this strain. Upon exit from the lag phase, the growth rate of both strains was similar, despite the extended lag phase in the H^+-ATPase mutant (Holyoak *et al.*, 1996), but the pH_i in the mutant was considerably less than in the parent. This would suggest that the mutant strain does not have to attain a certain value of pH_i similar to that in the parent before outgrowth from lag can occur. Therefore, it would seem that another physiological consequence of reduced-expression of the membrane H^+-ATPase other than reduced pH_i accounts for the extended lag phase in this strain.

It is generally accepted that low cytoplasmic pH is detrimental to the microbial cell, causing disruption to many critical physiological functions, such as, the regulation of key metabolic enzymes and cell division. Therefore, it is not surprising that a number of studies have implied that inhibition of growth by a number of stress factors could be due to disruption of pH_i homeostasis. For example, such stresses as heat (Weitzel *et al.*, 1987 and Coote *et al.*, 1994), low pH (O'Hara *et al.*, 1989), ethanol (Cartwright *et al.*, 1986) and weak acids (Salmond *et al.*, 1984 and Carmelo *et al.*, 1997) have all been attributed.

Many studies have suggested that the principal inhibitory action of weak acids is reduction in pH_i (Booth & Kroll, 1989, Salmond *et al.*, 1984 and Cole & Keenan, 1987). However, exactly why reduction of pH_i inhibits growth is less clear and a number of hypotheses have been proposed. Krebs *et al.* (1983) concluded that reduction of pH_i in yeast induced by exposure to benzoic acid resulted in the direct inhibition of phosphofructokinase which reduced glycolytic flux, energy generation and, ultimately, growth. Another proposed mechanism is that weak acid induced intracellular acidification results in the elimination of the proton gradient across the membrane and the inhibition of essential nutrient uptake, such as amino acid transport (Freese *et al.*, 1973). Despite these studies, the role of pH_i disruption in growth inhibition by weak acids remains unclear.

3.3.4. Comparison of the inhibitory effects of two different antifungal actives; sorbic acid and amphotericin B

Unlike the weak acids, the inhibitory action of amphotericin B is believed to be due to gross membrane disruption and channel formation, causing significant permeability of the yeast plasma membrane to protons (Palacios *et al.*, 1978). To determine the importance of membrane disruption and the effects upon pH_i homeostasis, the actives amphotericin and sorbic acid were compared.

In this study the results showed that the inhibitory activity of sorbic acid was dependent upon two factors. Firstly, the amount of growth inhibition increased as the concentration of membrane permeable undissociated weak acid increased (Figure 3.10 a)), thus confirming the observations of previous studies (Salmond *et al.,* 1984 and Holyoak, *et al.,* 1996). Secondly, the amount of growth inhibition was greater at lower culture pH values despite exposure to a constant concentration of undissociated sorbic acid (Figure 3.12 a)). Furthermore, the results in this study demonstrate that sorbic acid can have a significant inhibitory effect on the growth of *S. cerevisiae* without any appreciable reduction in pH_i (Figures 3.11 and 3.12 a)). Therefore, if direct pH_i reduction is not responsible for the inhibitory action of sorbic acid in yeast then there must be an alternative explanation.

In order to gain further understanding into the potential role of pH_i in growth inhibition, a comparison was performed of the inhibitory kinetics obtained with sorbic acid to those induced by exposure to amphotericin B. Despite inhibiting growth with similar kinetics to sorbic acid, the inhibitory activity of amphotericin B did correlate with reduction in pH_i at the lower pH values (Figures 3.10 b) and 3.11). Amphotericin inhibition was also highly dependent on the external culture pH (Figures 3.10 b), 3.11 and 3.12)), as all these results show the lower the external pH, the greater the inhibition of growth and the greater the decline in pH_i.

Furthermore, exposure to a constant concentration of amphotericin B at decreasing pH values resulted in alteration of membrane permeability leading to increasing proton influx into the cell as the external pH dropped (Figure 3.13). Therefore, the observed inhibitory effect of amphotericin B is consistent with gross membrane disruption (Palacios & Serrano, 1978), or proton-uncoupling, leading to growth

inhibition due to proton influx and decline in pH_i. Hence, the observations of greater inhibition of growth at lower culture pH values. Exposure to a constant concentration of undissociated sorbic acid had the same effect on membrane permeability as amphotericin B except little change in pH_i was observed (Figure 3.13).

Although large reductions in pH_i in cells resupended in growth media were not detected on exposure to sorbic acid using this studies methodology, other studies have reported sorbic acid induced decline in pH_i in yeast (Cole & Keenan, 1987) and *Escherichia coli* (Salmond *et al.,* 1984) at similar pH values. However, in these studies, pH_i was measured by a method based upon the distribution of radio-labelled weak acids, which involved centrifugation steps and measurement of pH_i in non-growing cells resuspended in buffers. Indeed, experiments in this study have shown that exposure of non-growing cells in citric / phosphate buffer to sorbic acid did result in a detectable reduction in pH_i (Figure 3.6 and 3.7). Nevertheless, when applying the same fluorometric method to determine pH_i in growing cells under physiological conditions in this study, the results do not show the same large sorbic acid induced reduction in pH_i.

A possible hypothesis to explain this could be that the sorbic acid would result in a detectable disruption in pH_i in the absence of any other homeostatic mechanism, e.g. induction of an energy-dependent stress response that could counteracts this detrimental effect.

There are two major pieces of evidence that support this hypothesis. Firstly, there is considerable evidence that sorbic acid does affect pH_i homeostasis due to the frequent observations that exposure to sorbic acid, and others organic acids, results in significant and rapid activation of the H^+-ATPase (Holyoak *et al.,* 1996, Piper *et al.,* 1997, Carmelo *et al.*, 1997 and Viegas *et al.,* 1991) which is used by growing yeast cells to regulate and maintain pH_i homeostasis. Secondly, the results in Figure 3.13 show that on exposure to sorbic acid, a membrane permeabilising effect is demonstrated, which is similar to that induced by exposure to a lower concentration of amphotericin B. This exposure thus resulted in an increasing proton influx across the membrane as the external pH declined and this effect we would expect to reduce the pH_i.

The available evidence supports a hypothesis such that the energy-state of the cells provides the underlying mechanism responding to protect the cells from the inhibitory effect of sorbic acid. The fact that disruption of pH_i homeostasis was not detected in this study does not necessarily mean that this was not taking place. The increased activation of the membrane H^+-ATPase, that is known to occur upon exposure to sorbic acid in growing cells (Holyoak *et al.*, 1996 and Piper *et al.*, 1997), may have resulted in the restoration of pH_i homeostasis such that any fluctuation became less detectable.

If this hypothesis is true, then how can the inhibitory effect of sorbic acid be explained?
It has been shown that coupling ATP hydrolysis to proton translocation can result in the membrane H^+-ATPase consuming up to 60% of total cellular ATP (Serrano, 1991). Thus importantly, this increased activity of the membrane H^+-ATPase is energetically expensive and such a depletion of ATP may restrict growth. Studies by Viegas *et al.* (1991) have shown that activation of the membrane H^+-ATPase by octanoic acid correlated with a decrease in the biomass yield and Holyoak *et al.* (1996) have shown that a glycolytic mutant, with approximately one-third the normal pyruvate kinase and phosphofructokinase activity and hence a reduced ability to generate ATP, was more sensitive to sorbic acid.

In this study it has been shown that on exposure to sorbic acid the intracellular ADP / ATP ratio increased (Figure 3.14). These results are in agreement with previous observations that also showed large reductions in the levels of intracellular ATP during growth in the presence of sorbic acid (Holyoak *et al.*, 1996). Thus, the inhibitory activity of sorbic acid can be explained as the induction of an energy-consuming response to disruption of pH_i homeostasis that reduces cellular energy pools to the extent that there is less free energy for growth and division.

Evidence also suggests that this energy-demanding stress could also be due to the induction of plasma membrane pumps for active extrusion of weak acids from the cell as first proposed by Warth (1977) and supported by data of Henriques *et al.* (1997) for benzoic acid. Indeed, a pump recently identified as the Pdr12 protein is induced by sorbic acid in *S. cerevisiae* (Piper *et al.*, 1998) and study of this deletion

mutant of Pdr12 has shown that active extrusion is important for resistance to sorbic acid (Piper *et al.,* 1998).

The results shown in Figure 3.14 also demonstrate that exposure to amphotericin B results in an increase in the ADP / ATP ratio. If, as this study has shown, this compound is acting to disrupt the plasma membrane, then perhaps the decline in pH_i observed occurs despite activation of the membrane H^+-ATPase, which is attempting to restore pH_i homeostasis. Supporting this, available evidence suggests amphotericin B forms stable membrane spanning channels (Bolard, 1986) that uncouple the membrane such that any increased activity of the H^+-ATPase attempting to restore pH_i homeostasis would be futile. Hence, exposure to increasing concentrations of amphotericin B results in a correlating decline in the pH_i and decreasing growth rate. In contrast, although this study has shown that sorbic acid does induce membrane disruption this does not uncouple the membrane sufficiently to prevent increased H^+-ATPase activity maintaining pH_i homeostasis.

To further discuss the membrane permeability effect of sorbic acid seen in Figure 3.13, the studies of Weber & de Bont (1996) have shown that actives that are highly lipophilic such as amphotericin and to a lesser extent sorbic acid, partitioning in the membrane causing an increase in membrane permeability. This in turn increases the flow of protons and other ions across the membrane, leading to the dissipation of the proton motive force and resulting in less effective energy transduction.
Other effects as reduction in activity of key membrane bound enzymes and loss of important metabolites can together cause major impact on cellular activity.

In summary, the results from this Chapter and other supporting evidence suggest that the growth inhibitory effect of sorbic acid on *S. cerevisiae* be due the disruption of a number of different cellular activities rather than one. The inhibition seen may arise from not only the effect of free proton and anions liberated upon the dissociation of the acid in the cell, but also from the membrane disruption effects discussed above. All of these resulting in different energy-consuming stress responses required to attempt to restore pH_i and cellular homeostasis.

Further work in this thesis will attempt to elucidate this model further to understand the exact molecular nature of this response.

Chapter 4 – The use of proteomic methodology to investigate the stress mechanisms in response to sorbic acid on the proteins of *S. cerevisiae*

4.1. Introduction

The world wide efforts to sequence different genomes began in the 1990's, amongst these the yeast, worm and fly sequences, as well as many prokaryotes have been completed (Dhand, 2000) and many of these genome projects are producing a wealth of information about the sequences of individual genes. For instance the completion of the analysis of the *Saccharomyces cerevisiae* genome sequence, well over 6000 predicted genes have been identified (Blomberg, 1997). The accumulation of vast amounts of DNA sequence, the scientific community is realising that completing the genomic sequence of an organism is not sufficient to elucidate biological function (Lockhart & Winzeler, 2000). Thus the focus has changed to not only identifying the structure and assigning function to the new genes but also to investigate interactions and organisation of genetic pathways that control the production of proteins as well the roles they in play in physiology of an organism using the discipline of functional genomics (Navarre *et al.*, 2002 and Dhand, 2000).

A cell or organism is dependent upon a multitude if metabolic and regulatory pathways to survive. But, because there is no strict relationship between genes and the protein complement (proteome) of a cell, both proteomics and genomics being complimentary to each other are needed to study both the genome, transcriptome (the mRNA complement) and the proteome simultaneously (Pandey & Mann, 2000).

4.1.1. Genomics and DNA arrays

To take advantage of the rapidly increasing body of sequence information and to interpret this for use in such areas as molecular biology and medicine, new investigative technologies have been developed. One such technology has been quoted as being the most powerful and versatile for genomics is that of high density

arrays of oligonucleotides or cDNA's and thus called DNA microarrays (Lockhart & Winzeler, 2000).

The technology has been applied to the monitoring of gene expression by measuring the transcribed mRNA. The collections of genes that are transcribed from the genomic DNA are sometimes referred to as the transcriptome, and are a major determinant of cellular phenotype and function. Like the proteome the transcriptome is highly dynamic and changes rapidly in response to cellular responses to environmental stimuli. Thus the technique enables a greater understanding of gene function, and ability to determine when, where and to what extent a gene is expressed due to a certain event or cellular phenotype. This knowledge being central to the understanding of biological activity of an encoded protein but also other multi gene expression patterns and the role they play in the broader picture of cellular function and biochemical pathways.

The basis of this technique consists of the hybridisation of a labelled unknown sample of RNA or DNA to an ordered array of immobilised DNA molecules of a known specific pattern which can be analysed or compared to a standard. This system has the potential of analysis of the genome and screening the molecular responses to a whole range of different environmental conditions and/ or chemicals in parallel (Hoheisel, 1997).

The first arrays used DNA fragments of often unknown sequence from either, cDNA, genomic DNA or plasmid libraries and the hybridised material was radio-labelled. However, recent developments mean that the use of glass substrates and fluorescent labels, along with the ability to deposit nucleic acids to very high densities, means that the transcriptome can be screened very rapidly. Arrays containing more than 250,000 different oligonucleotide probes or 10,000 different cDNA's per square centimetre can be routinely produced microarrays (Lockhart & Winzeler, 2000), however, many groups use a far less dense array often containing 10,000 genes across the whole slide (Pennington & Dunn, 2001).

This technique thus now allows the large scale screen of the whole genome for changes in transcriptome events, ensuring a complete unbiased view of the cellular response. This method is far simpler to perform, is highly sensitive and gives high

throughput of samples compared to protein based approaches (Lockhart & Winzeler, 2000). However, the drawbacks of this methodology are that mRNA abundance does not always correlate to protein abundance, the sensitivity and range of existing technologies are such that the lowest abundance mRNA's are not readily measurable alongside the more abundant and the activity of proteins encoded by mRNA's is regulated at several levels beyond protein expression, i.e. post translational modifications. Furthermore, for non-model organisms the availability of such arrays prevents such technologies to be used (Pennington & Dunn, 2001). Thus there is a need for a complementary approach between DNA microarray techniques and proteomics to provide a complete picture (Pennington & Dunn, 2001 and Lockhart & Winzeler, 2000).

4.1.2. The proteome and proteomic technology

The proteome is effectively the total complement of proteins encoded by the genome which are produced in a cascade of regulatory events. The genome is comparatively static whilst the proteome like the transcriptome, is also a highly dynamic entity, as at any given moment the protein content of the cell may change due to influences such as from, the environment, stress, and physiological state of the cell, thus the proteome represents the expression of the genome at a certain time point (Rabilloud *et al.*, 2000). Any protein modifications are not apparent from the DNA sequence, such as isoforms or post translational modifications, and thus can only de determined by proteomic methodologies (Pandey & Mann, 2000).

Proteomics is the large scale study of proteins usually using biochemical methods and can be divided into three main areas; protein micro-characterisation for large scale identification of proteins and their post-translational modifications, differential display comparing proteins levels between two conditions and protein-protein interactions (Pandey & Mann, 2000). This study utilises the proteomic techniques described in the second area to compare changes due to a change in the environment.

Proteomics consists of two independent phases, the separation of the bio-molecules of interest and their molecular characterisation using a high resolution two-dimensional polyacrylamide gel electrophoresis technique (2D-PAGE) to chemically

resolve the proteins (first described by O'Farrell, 1975). This technique combines the separation of proteins according to their charge or isoelectric point (pI) by isoelectric focussing (IEF) in the first dimension and then according to size or molecular weight (MW) by SDS-PAGE in the second dimension. This technique enables reproducible results to be produced and is the only one that currently has a high enough resolution to separate the potentially thousands of proteins present in a cell (Rabilloud *et al.*, 2001).

Proteomic technology like genomic technology has made huge advances since the early 1970's when it first came into existence. However, it was not till the 1990's before it was possible to resolve complex mixtures of cellular proteins and also to make identifications of key proteins of interest. The development of biological mass spectrometry to analyse such proteins removed these limitations and now offer a powerful technique for the investigation of cellular expression in many organisms (Boucherie *et al.*, 1995). This coupled with the publishing of numerous genome sequence databases has enable rapid advances in identifying key proteins with genes thus the term functional genomics now covers this area (Pandey & Mann, 2000).

O'Farrel (1975) first reported the use of this technique to study *E. coli*, and showed that complex mixtures of cellular proteins could be separated out, providing a global overview of cellular expression. Many others have since used this technique, such as, Cash (1991) to study the structure and replication within viruses and bacteria and Boucherie *et al.* (1995) to study *S. cerevisiae*. They have showed that many novel proteins could be identified and in particular, those that were involved in heat shock and cellular stress responses in yeast, furthermore, Boucherie *et al.* (1995) demonstrated the importance of this technique to link proteins to functionality.

The methodology since described by researchers has been improved to give good reproducibility of the separation technology for IEF in the form of immobilised gradients. By running all comparable samples together, gel to gel variation can be minimised. However, until recently, it was found to be almost impossible to analyse all of the separated proteins on a large 2D gel (Cash, 1991). However, the advancement of proteomics now also uses a combination of other sophisticated techniques including the above described 2D electrophoresis with image analysis,

mass spectrometry, amino acid sequencing and bio-informatics to resolve, quantify and characterise proteins. Further advances in computer software for construction of polypeptide maps and comparison by image analysis have allowed far faster determination of protein differences due to a specific environment, both qualitatively and quantitatively. Plus the ability to analyse large numbers of gels simultaneously ensures that results found are reproducible and robust (Pennington & Dunn, 2001).

The existence of an open reading frame (ORF) in genomic data does not necessarily imply the existence of a functional gene and despite the advances of bioinformatics it is still difficult to predict genes accurately from genomic data. Thus using proteomics to verify a gene product is the first step in annotating the genome (Pandey & Mann, 2000). Furthermore, a proteomic analysis can provide information on, if and when predicted gene products are translated, but also the relative concentration of these. In addition, the extent of post translational modification e.g. proteolytic cleavage, glycosylation and phosphorylation of proteins can be determined by this technique, all of which can have a dramatic effect on protein activity and/ or function (Kannicht, 2002). Proteomics has also allowed the study of multigenic responses such as, protein expressed during cell cycles, or during stress treatments, which is of interest and importance for the benefits of this study, as none of the above can be accurately predicted from DNA sequence alone (Wasinger *et al.*, 1995).

4.1.2.1. The disadvantages and short comings of proteomics

Boucherie *et al.* (1995) discussed the difficulties found when constructing a gene to protein index and the comparison of results from different gels run at different times. They concluded that there were problems in pattern comparisons that were as a result of experimental procedural variations and that any changes to experimental protocols resulted in differences in the relative mobility of proteins in 2D-PAGE gels. Thus comparison of pattern differences across gels from the same lab let alone across different labs was a great challenge. Blomberg (1997) also stresses that not only the standardisation of equipment, but also of growth conditions and extraction methodology should be employed to ensure confidence in cross proteomic gel comparisons. The use of key anchor proteins within a proteomic gel has helped to provide landmarks to establish correlation's to the positioning of different proteins.

However, Boucherie *et al.* (1995) go onto warn that when investigating proteins of low abundance this correlation technique could be hazardous and numerous repeats are required to ensure a certain identification.

Navarre *et al.* (1995) describes further the issues and problems when using proteomics for investigating plasma membrane protein expression. The results by many researchers have so far shown that at best only about 1,200 proteins can be separated by 2D-PAGE, which corresponds to about one quarter of the proteins reported. It seems that issues that many with low abundance proteins can not be detected and often these proteins are barely visible if not at all.

Another such issue is that of protein solubility in the cellular extract when preparing total protein preparations and studies have shown that many of the proteins that are absent from extracts are of a more hydrophobic nature and of the proteins present in the extract, none were shown to have trans-membrane domains (which represent about 30% of the total proteins). Adaptation of methods using detergents and organic solvents has given great improvements in the solubilisation of membrane proteins and thus better separation by 2D-PAGE (Navarre *et al.,* 1995 and further described by Rabilloud *et al.,* 2001).

4.1.3. The use of pulse labelling within proteomics

Many traditional 2D-PAGE methods using standard organic dye staining methods suffer from low-resolution when detecting proteins of low copy numbers. Thus to improve such resolution many researchers have used *in vivo* selective incorporation of radio-labelled amino acids (using such isotopes as ^{3}H, ^{14}C and ^{35}S). Maillet *et al.* (1996) showed that using such a technique gave far better resolution and identified 79 new protein spots within *Saccharomyces cerevisiae.* The majority of researchers still prefer non radioactive methods, for instance using silver stain for detection of proteins on 2D gels due to its sensitivity. Silver staining also has the advantages over radio-labelling as the uptake of the stain is not dependent upon metabolic activity resulting in detection of all proteins. However, the advantages of high sensitivity and advances of autoradiography and the availability of a variety of labelled amino acids

means that radio-labelling is becoming more widely used (Humphrey-Smith *et al.*, 1997).

Within this study the radio-labelled amino acid ^{35}S-methionine was used to pulse label *Saccharomyces cerevisiae*. Using synthetic growth media containing a full complement of amino acids minus methionine ensured the radioisotopes uptake by the yeast cell and thus incorporated within the cellular protein. So that the methionine would not be converted into another amino acid and the label lost, all other amino acids required by the yeast cell were provided in the media. The benefits of such a method allow a non-invasive labelling technique that ensures the cells are as far as possible to be present in their natural state.

4.1.4. Peptide-mass fingerprinting and protein analysis

There are a wide variety of techniques available to characterise proteins, whilst 2D-PAGE can provide the approximate isoelectric point (pI) and molecular weight (MW), this information in itself is not sufficient to identify most proteins. Thus one of the most commonly used methods to improve identification is peptide-mass fingerprinting using biological mass spectrometry (MS) (Chambers *et al.*, 2000).

Biological MS has become a crucial tool for rapid protein and peptide structural analysis and the widespread use of MS is a reflection of its importance over other techniques. The development of this technique was a significant breakthrough to identify gel separated proteins, as mass spectrometry essentially replaced Edman degradation (Pandey & Mann, 2000). In addition it has been shown to be sensitive, can deal with complex protein mixtures and has the potential for high throughput (Jensen *et al.*, 1997). There are two MS techniques that have become preferred methods for ionisation of peptides and proteins, these being electrospray ionisation (ESI) and matrix-assisted laser desorption / ionisation (MALDI) with time of flight tube (TOF). Both techniques give best results with salt and detergent free samples, but the MALDI is more tolerant and robust of the two with sample contaminants, but is also more sensitive (Rabilloud *et al.*, 2001).

The proteins once identified are excised from the original stained two-dimensional gel and are then digested using a sequence-specific proteolytic enzyme such as

trypsin, which cleaves the C-terminal to the amino acids arginine and lysine. This produces a set of fragments unique to that given protein, i.e. its own unique "mass fingerprint" or peptide mass map. The reason for analysing peptides rather than whole proteins is that proteins are difficult to elute and analyse by this technique.
The peptide fragments are then analysed in the mass spectrometer, such as in the above mentioned MALDI-TOF, to identify its origin (Chambers *et al.*, 2000). Often a fingerprint is produced to such a high degree of specificity that it is possible to identify the protein from this information alone. However, using this information, along with 2D-PAGE migration patterns, a high degree of certainty on identification can be given.

The data from this analysis is then evaluated using a peptide-mass fingerprinting tool such as, MS-FIT, SwissProt or Mascot, which contains information on expected mass fingerprints for every protein in a sequence database and searches are able to be conducted through various systems that are available on the internet. In the case for this study a few different databases were used, which took the resultant peptide analyses and "fitted" them to a protein sequence, thus suggesting the identity of a particular protein that matched the entered sequence (Jensen *et al.*, 1997). By using different protein databases e.g. MASCOT, a consensus in the results for a set of fingerprints can be determined. The MOWSE score that each database calculates is a probability value on how good a match the sequence is with the identity for a given protein. In general a value greater than 53 is deemed significant ($p<0.05$). Thus if this value is high in both databases then reassurance can be found that the match is a good one and the identity of this protein to the fingerprint is true. By using this data along with the score of the hit, plus the predicted molecular weight and isoelectric point (pI) of the protein, the experimental molecular weight and pI of the excised protein from the original gel can be compared to give further reassurance of a match.

The peptide mass fingerprinting studies and database searching, that are discussed within this study were performed at the University of Aberdeen by L. Laurie and de Nobel *et al.* (2001), describes further the methodology used for this work.

4.1.5. Potential applications of proteomics and the future

As the code for the human genome becomes increasingly available, so the use of proteomics to study human disease has increased. For instance, the use of proteomic analysis has been applied to the research of heart disease and the protein expression that underlies it and also to investigate bladder cancer at a molecular level. Recent advances in both genomics and proteomics have provided an opportunity to expand the range of potential drug targets and have facilitated a shift from direct antimicrobial screening towards target based programs. Such technologies have begun to revolutionise the pharmaceutical industry's capability for antimicrobial drug hunting. Thus hopefully solving some of the problems with increasing resistance of microbes to existing antibiotics, but also with the increase in the number microbes which have become pathogens, there is clearly a need for new drugs with new mechanisms. These studies have been utilising genomics in two areas, the investigation of specific genes in defined tissues and organs during an infection and functional analysis of antimicrobials (Rosamund *et al.,* 2000).

Many of the current applications of proteomics have been used in microbiological research to link genomes and proteomes of a variety of bacterial and yeast species. Research in particular with yeast has shown that these simple organisms can be used as very useful models for the integration of genomic and proteomic information in eukaryotic cells.

Recent developments in the automation of proteomic systems have allowed the possibility of coupling spot-excising robots to image analysis software. This allows what was a slow and laborious study of one gel to such a system that is capable of rapidly removing thousands of protein spots from multiple gels and transferring to an automated protein digestion work station, thus becoming essential for high throughput proteomics and analysis of large numbers of separated spots (Chambers *et al.*, 2000). In conjunction with this, there have been major advances in image analysis software packages enabling rapid and precise determination of proteins whose expression has been altered (Cutler, 2003). Even with the above advances, 2D-PAGE and MS still have their disadvantages both are labour intensive and require a high degree of skill to operate them.

Currently new methods are under development to possibly overcome some of these disadvantages. One such method is that of micro-channel-based separation, which employs rapid resolution of small amounts of nucleic acid mixtures. This technique uses SDS capillary gel electrophoresis of proteins giving high resolution and speed to protein separation. The system then uses the micro-channels photo etched upon a chip and the proteins are analysed by labelling with fluorescein. Detection then follows the electrophoresis by laser-induced fluorescence (Chambers *et al.*, 2000).

Another technique under development which has similarities to DNA Microarray is that of 'Protein Chips or array'. This technique uses a variety of 'bait' proteins such as antibodies in an array format bound to a surface to form a chip which can scan a cell in two different states using fluorescent probes to detect the differences (Pandey & Mann, 2000). Whilst the technology for DNA arrays is highly developed, the components for protein arrays are less so. Unlike DNA, proteins do not react by simple linear hybridisations and their properties are altered markedly by different environments. Thus any possible changes in protein conformation will affect its affinity to immobilise onto a solid surface. Despite these significant technical challenges, protein arrays could offer the high-throughput assays and a level of automation that existing 2D-PAGE can not achieve (Cutler, 2003).

There is a strong synergistic relationship between proteomics and genomics as the two disciplines investigate the molecular organisation of the cell at complementary levels (proteins and genes) and each discipline increases the effectiveness of the other (Pennington & Dunn, 2001). This study used only the described proteomic techniques of 2D-PAGE using both traditional staining and pulse labelling with ^{35}S methionine to identify cytosolic proteins with altered expression due the to exposure to sorbic acid. The resultant proteins that have changed expression in response to the weak acid stress would hopefully be identified as further key stress elements involved in stress resistance.

4.2. Results

4.2.1. Determination of protein concentration and radio-label incorporation of the total protein preparations of *S. cerevisiae*

A combination of one-dimensional SDS-acrylamide gels, Biorad protein assay and scintillation counting were used to determine the relative protein concentrations of the total protein preparations of *S. cerevisiae.* The results of these techniques also gave an indication of the level of purity of the extraction by the clarity and resolution of the separated bands.

The protein concentrations of the total protein preparations for both the non-labelled and pulse labelled control and sorbic acid stressed *S. cerevisiae* samples were determined using the Biorad protein assay and the amount of radio-label counts incorporated were determined by scintillation counting.
The results for these assays are shown in Table 4.1.

Table 4.1
The calculated total protein concentration for the four pulse-labelled total protein samples using the Biorad protein assay and resultant data from scintillation counter

Sample Name	Biorad Assay Protein Concentration	Average CPM per 20µl of sample	Volume of sample for 300,000 CPM
Control 1 hour	100 $\mu g ml^{-1}$	607 742	9.87 µl
Control 3 hour	128 $\mu g ml^{-1}$	631 756	9.49 µl
Sorbic Acid 1 hour	280 $\mu g ml^{-1}$	1 029 204	5.83 µl
Sorbic Acid 3 hour	135 $\mu g ml^{-1}$	1 806 560	3.32 µl

Previous experimentation of loading different radioactive levels onto the 2D-PAGE showed that a level of 300,000 CPM per gel was required to provide good resolution of the protein spots, when exposing the gel to autoradiographic film (results not shown). Thus using the scintillation counts data, the volumes of sample required for 300,000 CPM are also shown in Table 4.1. These values enabled the same amount of protein or radioactive counts per min (CPM) to be added for each sample, so that the spot sizes on both the autographs and the stained gels could be compared.

To check that the calculated dilution values for each of the samples was giving comparable sample concentrations for either the protein concentration or counts, the samples were separated in a one-dimensional SDS-acrylamide gel. The results showed that the intensity of the bands in the autograph were of a similar level, thus demonstrating that the same amount of counts for control 1 hour (C1), control 3 hour (C3) and sorbic acid 3 hour (S3) had been achieved (results not shown). However, for the sorbic acid 1 hour (S1) the bands were of a lower intensity and thus the sample was counted again in the scintillation counter to ensure that the same amount of counts was loaded. Furthermore, the intensity of the bands of the same gel when stained gave a similar result (results not shown).

4.2.2. Two-dimensional gel electrophoresis to separate ^{35}S-methionine pulse labelled total protein preparations of *S. cerevisiae*

Total protein samples extracted from ^{35}S-methionine pulse labelled 1 hour and 3 hour control and sorbic acid stressed *S. cerevisiae* were extracted into Laemmli buffer and separated by two-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Two different sample amounts were used for the four samples mentioned above, these being, 300,000 CPM of radio-labelled protein and 15 µg of total protein which were loaded onto the 2D-PAGE gels. Each gel was run in duplicate and compared to ensure no overriding differences were seen.

Once the stained gels had been exposed to the autoradiographic films, the gel was over lade with the film and the molecular weight markers from the gel were marked onto each autoradiograph. In addition the direction of the isoelectric point positions (pH 4 and 7) were also marked for reference.

Only two of the possible 16 gels are represented within this results section, the scanned images of the control (Figure 4.1) and sorbic acid stressed (Figure 4.2) sample pair from 1 hour pulse labelling with 15 µg protein loaded onto each.

4.2.3. Computer analysis of the 2D-PAGE autoradiographs for the radio-labelled total protein preparations of *S. cerevisiae*

Each control and sorbic acid gel pairing was analysed by pairing spots both manually then automatically via the computer analysis package 2D-Elite till the majority of spots were paired. Certain key anchor spots or landmarks were identified to aid this process, which have been shown not to vary greatly in different stress conditions and thus helped the computer to form anchors across the gels (Pennington & Dunn, 2001).

The set of gels for the four samples containing 300,000 CPM of labelled protein and likewise for the gel set containing 15 µg of protein, a control and a sorbic acid gel 1 hour and similarly for 3 hours were matched together to form a pair. This allowed differences in protein expression to be identified as a result of the sorbic acid stress using the control as the reference point. This matching process was repeated for all of the gel pairs, 1 hour and 3 hour labelling times for both 300,000 CPM and 15 µg protein amounts per gel, producing a whole series of matches.

Each spot on each gel was uniquely numbered, which enabled it to be identified in any further matches and a spot that had been matched between a control and sorbic acid gel pair was given a two number reference (i.e. a combination of numbers from each gel).

Figure 4.3 shows an example of one of the computer analysis images produced for a pair of gels. The image shows in particular the match between the control and sorbic acid samples after 1 hour of pulse labelling with 15 µg of protein per gel. For each analysis the control spots were circled in blue and the sorbic acid stress sample spots in red.

For each spot the computer calculated the relative pixel intensity from the area within the circle, providing a quantitative value for each identified protein. The pixel intensities for each control spot were then compared against the equivalent matched spots on the sorbic acid gel. The relative increase or decrease in pixel intensity was determined using the control as a reference point, and any increase or decrease over 5 times that of the control was deemed to be of a significant difference and thus the identified spot was short-listed for further analysis.

The short-listed candidates were further cross compared to identify any spots that showed a uniform pattern of increased or decreased expression across all of the gel pairs, these were then short-listed further for excision and identification by mass spectrometry. To locate the candidates each autoradiograph was over laid against the original stained gel and the molecular weights and pI co-ordinates calculated (data represented in Table 4.2).

The identified spots of interest are depicted further in Figures 4.4 to Figure 4.11 showing an enlarged image comparing the control gel to the sorbic acid gel in each case.

As an indication of success of the separation technique for the proteins the total number of proteins separated and detected were counted for each gel. This number ranged from 700 to 1000 proteins. This is probably the best that can be expected as the most that can be usually separated by this type of technique using such equipment, a maximum of 1,200 can be obtained (Navarre *et al.*, 1995).

Table 4.2

Comparison of spots identified for further investigation, including calculated MW and pI from the 2D-PAGE gels

Spot Ref. Number	MW / pI value	Expression change due to sorbic acid (SA)
48	60,000 / 6.7	Switched on for both 1 and 3 hour SA gels
68	49,000 / 6.7	6 times increase in expression for both 1 and 3 hour SA gels
86	49,000 / 5.1	9 times increase in expression from 1 hour to 3 hour
144	38,000 / 6.8	21 times increase in expression for both 1 and 3 hour SA gels
185	33,000 / 6.8	Switched on for both 1 and 3 hour SA gels
187	37,000 / 5.1	Switched on for both 1 and 3 hour SA gels
301	19,900 / 6.6	Switched on for both 1 and 3 hour SA gels
315	17,000 / 6.8	Switched on 1hour but not present on 3 hour SA gel

The spot number used as the reference number is that from the sorbic acid 1 hour 15 µg gel.

4.2.4. Identification of spots by molecular fingerprinting by mass spectrometry

The sorbic acid 1 hour non-radio labelled Gelcode stained gel with 15 µg of protein per gel was used to excise from it the identified proteins highlighted in Table 4.2. The autoradiographic films were overlaid onto an equivalent non radio-labelled stained gel and the molecular weight and pI co-ordinates were also used to elucidate the exact location of each protein of interest. This was performed to ensure that any identified proteins excised from the gels were unlabelled for peptide mass fingerprinting.

The tryptic-digested fragments were sorted by the MALDI-TOF spectrophotometer and the results produced by the software into a graphical printout for analysis. An example of one of these analyses is shown in Figure 4.12, which depicts the output from the computer analysis software for the digested protein spot number 68. The size and mass of the fragment peaks are marked on the print out, forming a unique fingerprint for that protein enabling its identification. The sets of fragment sizes for each of the different proteins were entered into the MASCOT database and matches sought against known protein peptide sequences from the identified yeast genome. The matches were each given a probability based Mowse score, with a score greater than 51 ($p<0.05$) deemed significant.

The results of the matches for all eight of the protein spots sent for identification are summarised in Table 4.3. The table contains the Mowse score for each excised protein spot and possible matches from the database. In addition the experimental MW and pI were compared to that reported in the literature (using MIPS, YPD and SGD databases and web-sites) in all cases the mature protein is quoted.

SGD database: www.stanford.edu/group/yeast_deletion_project/deletions3.html
MIPS database: www.mips.gsf.de/genre/proj/yeast
YPD database: www.proteome.com

The proteins identified in Table 4.3 were further short-listed as a result of relevance to having possible roles within the yeast stress response.

Table 4.3

Results from the MASCOT database for excised protein spots with cross-referencing of theoretical and experimental MW and pI of identified proteins

Spot Number	Mowse Score	Protein name / ORF name	MW / pI from gel	MW / pI from refs.	Comments
48	67	Ach1p / YBL015W	60,000 / 6.7	58,500 / 6.5	Acetyl CoA hydrolase - hydrolyses Acetyl CoA chains
48	53	Ero1p / YML130C	60,000 / 6.7	65,000 / 5.6	Ero1p - catalyses disulphide bond formation
68	302	Cys4p / YGR155W	49,000 / 6.7	55,000 / 6.5	Cystathione beta-synthase - converts serine and homocysteine to cystathione
86	92	Atp2p / YJR121W	49,000 / 5.1	51,109 / 5.4	ATP synthase beta chain for proton transporting
86	51	Hsp26p / YBR072W	49,000 / 5.1	25,000 / 5.3	Heat shock protein 26 - involved with stress responses
144	129	Ilv5p / YLR355C	38,000 / 6.8	44,300 / 9.1	Ketol-acid reductoisomerase catalyses reactions for isoleucine and valine biosynthesis
144	83	Adh1p / YOL086C	38,000 / 6.8	36,800 / 6.2	Alcohol dehydrogenase 1
185	107	Tdh2p / YJR009C	33,000 / 6.8	35,800 / 6.7	Glyceraldehyde 3 phosphate dehydrogenase – glycolysis enzyme
187	84	Hcr1p / YLR192C	37,000 / 5.1	30,000 / 5.1	Protein of no known function, similar to a translation initiation factor eIF3
301	58	Tdh3p / YGR192C	19,900 / 6.6	35,800 / 6.5	Glyceraldehyde 3 phosphate dehydrogenase – glycolysis enzyme
315	122	Cph1p / YDR155C	17,000 / 6.8	17,288 / 6.7	Cycophilin peptidyl-prolyl cis-trans isomerase – involved with stress response

4.2.5. Comparative analysis of the proteins identified from this study to those from other studies as a result of weak acid stress

The proteins identified from this study as a result of the sorbate stress were cross-compared with the results from other types of analysis looking for changes in either the phenotypes or genotypes of yeast. As techniques have developed and the yeast genome sequenced, it has become increasingly possible to conduct large-scale analysis of the effect of such weak acid stresses across the whole genome or proteome.

The work of Schuller *et al.* (2004) used both global phenotypic analysis of the EUROSCARF collection and global transcriptome microarray analysis to fully assess the effect of weak acid stress on the cell.
Other studies by Mollapour *et al.* (2004) used a similar approach to Schuller *et al.* (2004) of phenotypic analysis but screened gene deletion mutants from a diploid background and both studies used yeast strains of a TRP^+ background.
Finally the work of de Nobel *et al.* (2001) was also compared who used a similar proteomic approach as this study using the same FY parents for the analysis, but also used microarrays to give further insight into the cellular response to sorbate stress.

Table 4.4 contains the summarised comparison of the findings of this study with those of the others studies quoted above.
The findings are discussed more fully in the conclusions.

Table 4.4

Comparison of results of identified proteins from this study with those of other published studies

Protein Name / ORF name	Comparison with published literature
Ach1p / YBL015W	Schuller *et al.*, 2004 using DNA Microarray – gene induced de Nobel *et al.*, 2001 using proteomics – upregulated protein
Ero1p / YML130C	Not identified in any other studies
Cys4p / YGR155W	Mollapour *et al.*, 2004 using phenotypic analysis of gene deletion mutants in diploid background
Atp2p / YJR121W	de Nobel *et al.*, 2001 using proteomics – upregulated protein
Hsp26p / YBR072W	Schuller *et al.*, 2004 using DNA Microarray – gene induced de Nobel *et al.*, 2001 using proteomics – upregulated protein de Nobel *et al.*, 2001 using DNA microarray – gene induction increased
Ilv5p / YLR355C	Schuller *et al.*, 2004 using DNA Microarray – gene repressed – complete opposite to finding of this study
Adh1p / YOL086C	Schuller *et al.*, 2004 using phenotypic analysis of gene deletion mutants in haploid background
Tdh2p / YJR009C	de Nobel *et al.*, 2001 using microarray – gene induction increased
Hcr1p / YLR192C	Not identified in any other studies
Tdh3p / YGR192C	Mollapour *et al.*, 2004 using phenotypic analysis of gene deletion mutants in diploid background
Cph1p / YDR155C	de Nobel *et al.*, 2001 using microarray – gene induction increased

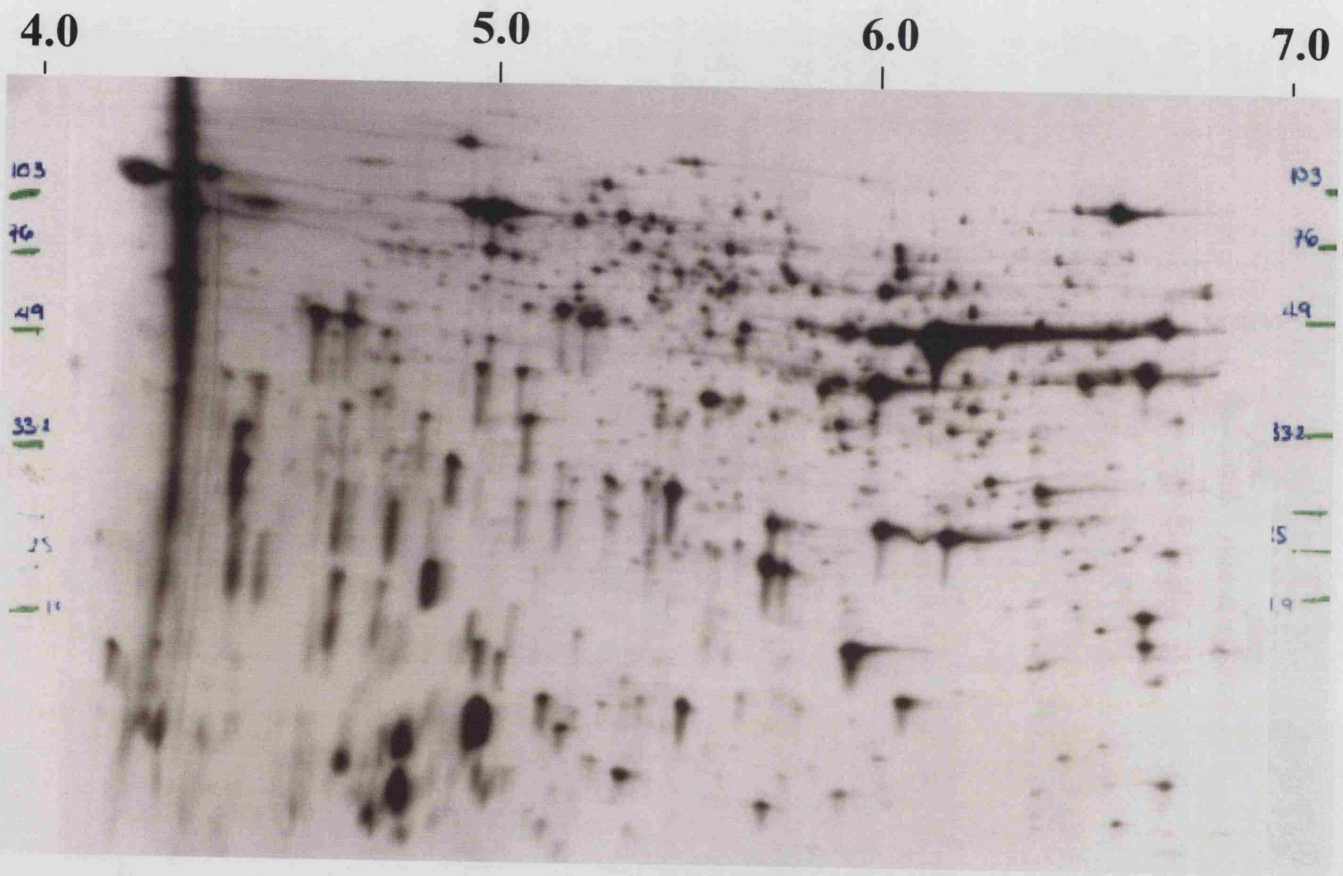


Figure 4.1

Two-dimensional SDS-PAGE gel autoradiograph loaded with 15 µg of a control protein sample of *S. cerevisiae* that has been radio-labelled for 1 hour with ^{35}S-methionine. Low range molecular markers (MW x 10^3) were marked along the sides of the gel and the pI scale from pH 4.0 to 7.0 marked along the top.

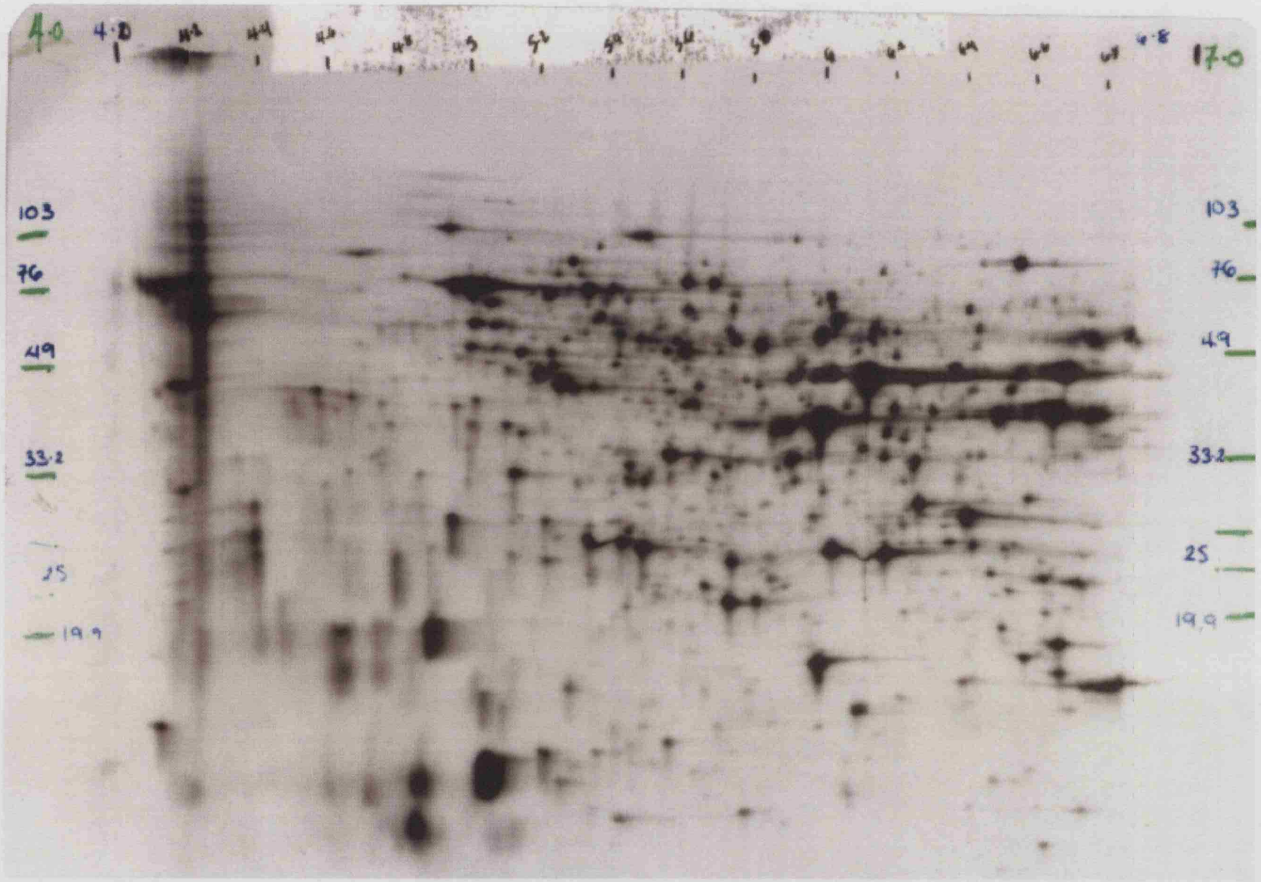


Figure 4.2

Two-dimensional SDS-PAGE gel autoradiograph loaded with 15 µg of a sorbic acid stressed protein sample of *S. cerevisiae* that has been radio-labelled for 1 hour with ^{35}S-methionine. Low range molecular markers (MW x 10^3) were marked along the sides of the gel and the pI scale from pH 4.0 to 7.0 marked along the top.

Figure 4.3

Computer image analysis from the 2D-ELITE software of the control and sorbic acid paired gels 1 hour of pulse labelling with 15 µg protein added per gel. The control spots are circled in blue and the sorbic acid stress sample spots are in red, with lines linking a paired match.

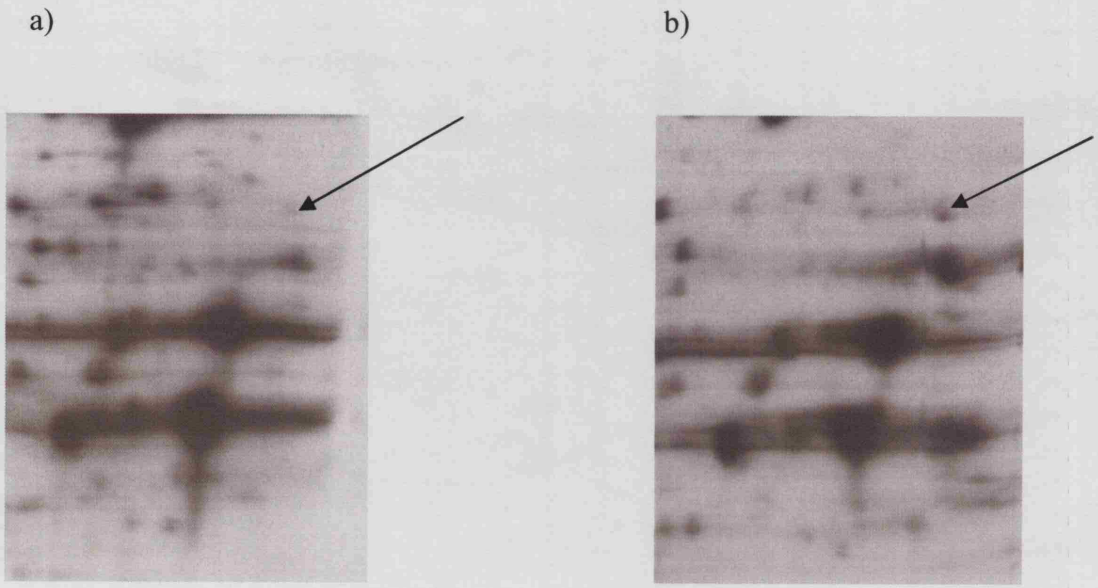


Figure 4.4 a) and b)

Enhanced gel image comparison of a) control and b) sorbic acid gels with the arrow identifying the position of spot number 48 on the sorbic acid 1 hour 15 µg gel and its comparative position of the control gel.

This sorbic acid stressed protein having been switched on compared to the control.

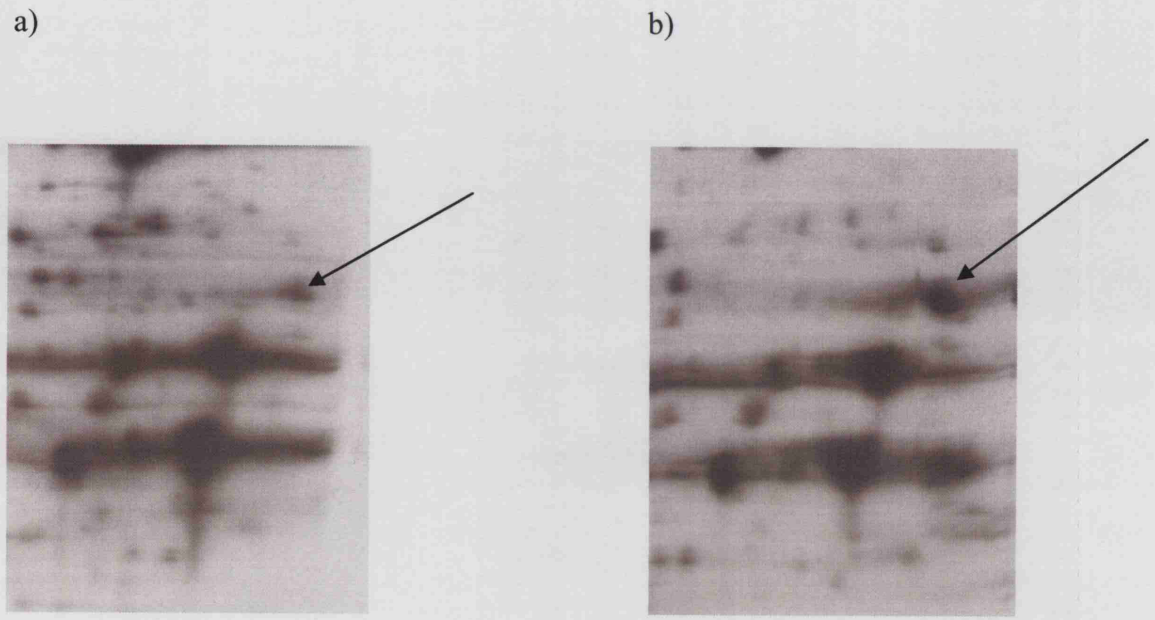


Figure 4.5 a) and b)

Enhanced gel image comparison of a) control and b) sorbic acid gels with the arrow identifying the position of spot number 68 on the sorbic acid 1 hour 15 µg gel and its comparative position of the control gel.

This sorbic acid stressed protein showing an increase in expression of over 6 times that of the control.

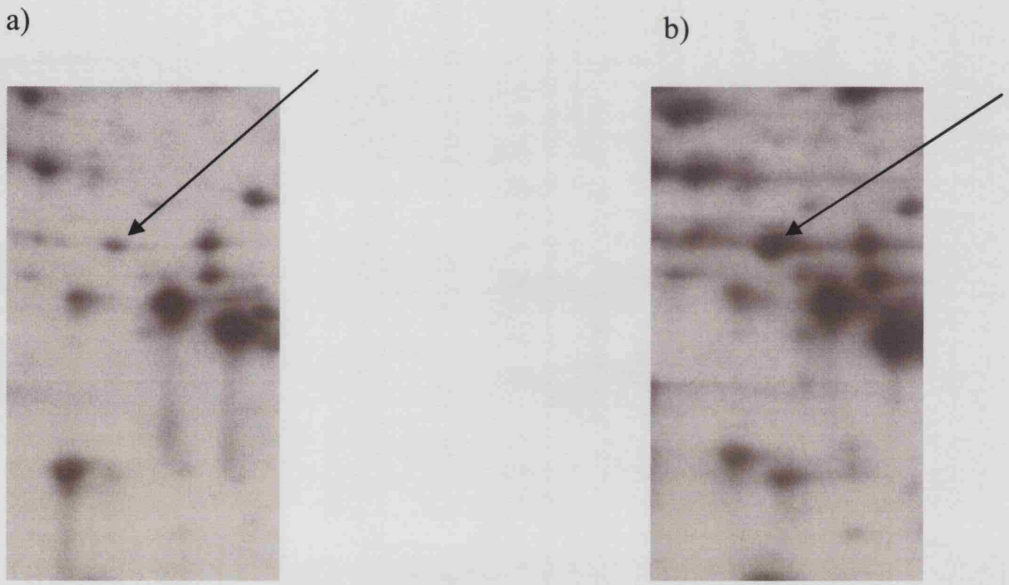


Figure 4.6 a) and b)

Enhanced gel image comparison of a) control and b) sorbic acid gels with the arrow identifying the position of spot number 86 on the sorbic acid 1 hour 15 µg gel and its comparative position of the control gel.

This sorbic acid stressed protein showing an increase in expression of over 9 times that of the control.

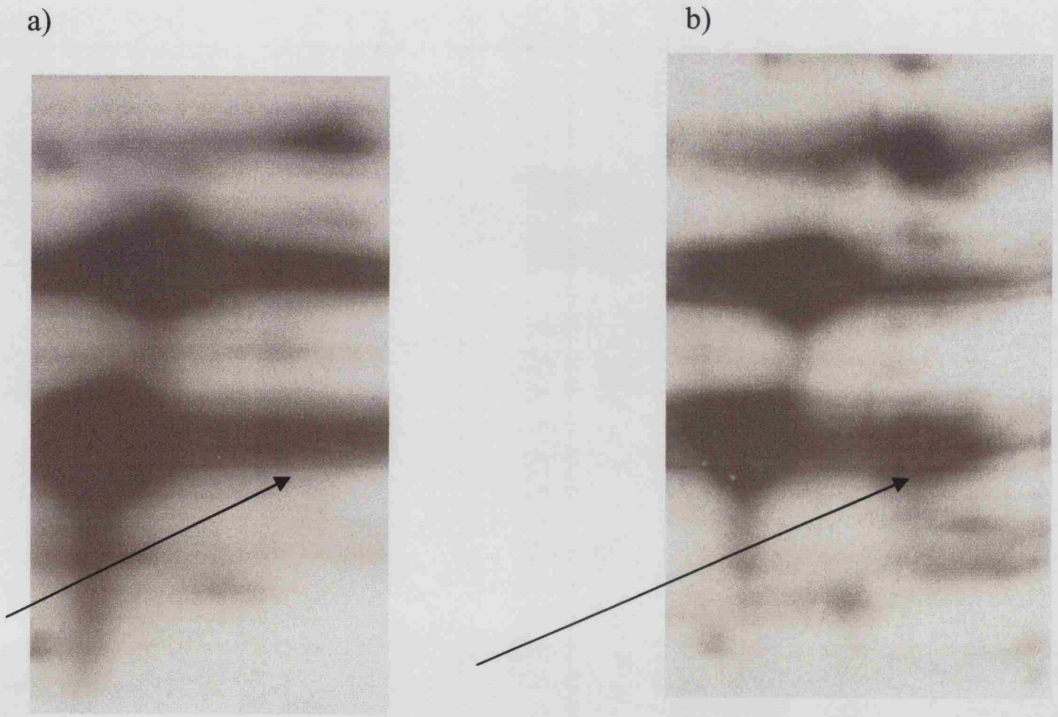


Figure 4.7 a) and b)

Enhanced gel image comparison of a) control and b) sorbic acid gels with the arrow identifying the position of spot number 144 on the sorbic acid 1 hour 15 µg gel and its comparative position of the control gel.

This sorbic acid stressed protein showing an increase in expression of over 21 times that of the control.

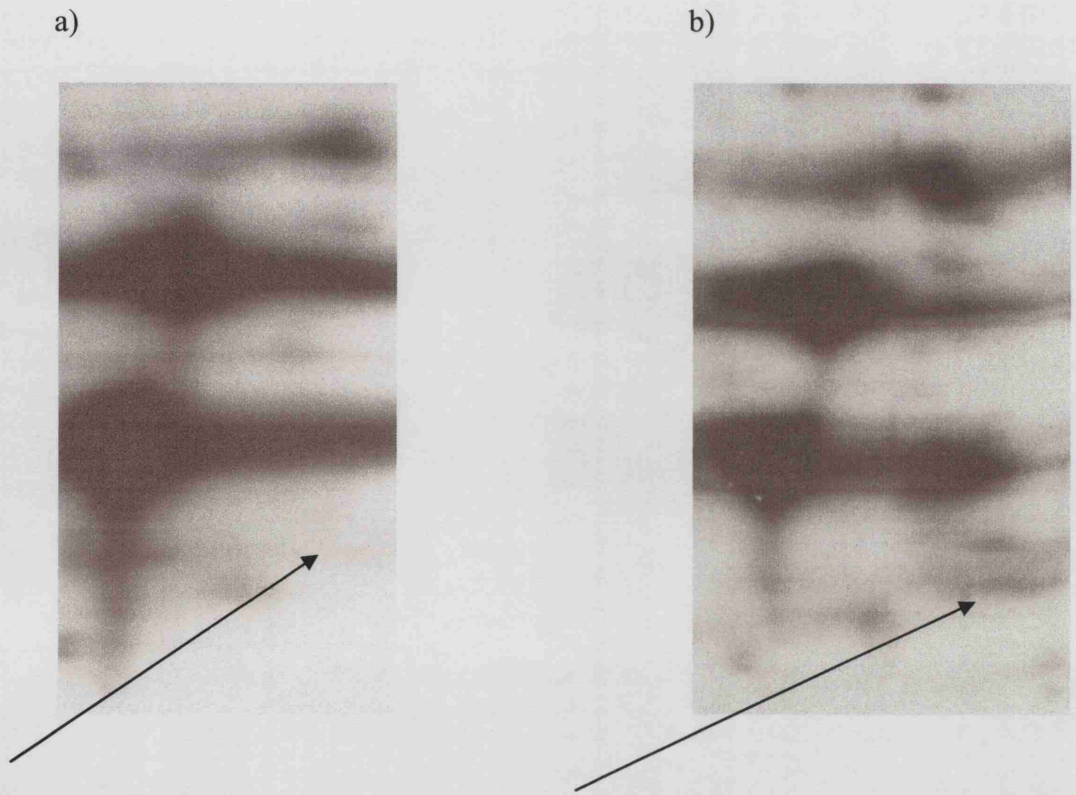


Figure 4.8 a) and b)

Enhanced gel image comparison of a) control and b) sorbic acid gels with the arrow identifying the position of spot number 185 on the sorbic acid 1 hour 15 µg gel and its comparative position of the control gel.

This sorbic acid stressed protein having been switched on compared to the control.

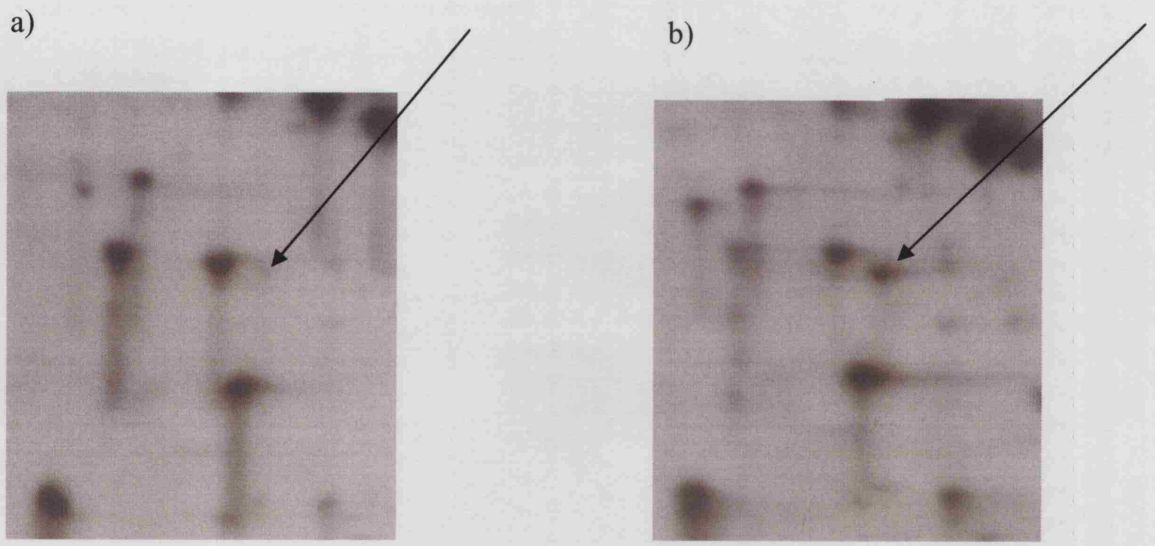


Figure 4.9 a) and b)

Enhanced gel image comparison of a) control and b) sorbic acid gels with the arrow identifying the position of spot number 187 on the sorbic acid 1 hour 15 µg gel and its comparative position of the control gel.

This sorbic acid stressed protein having been switched on compared to the control.

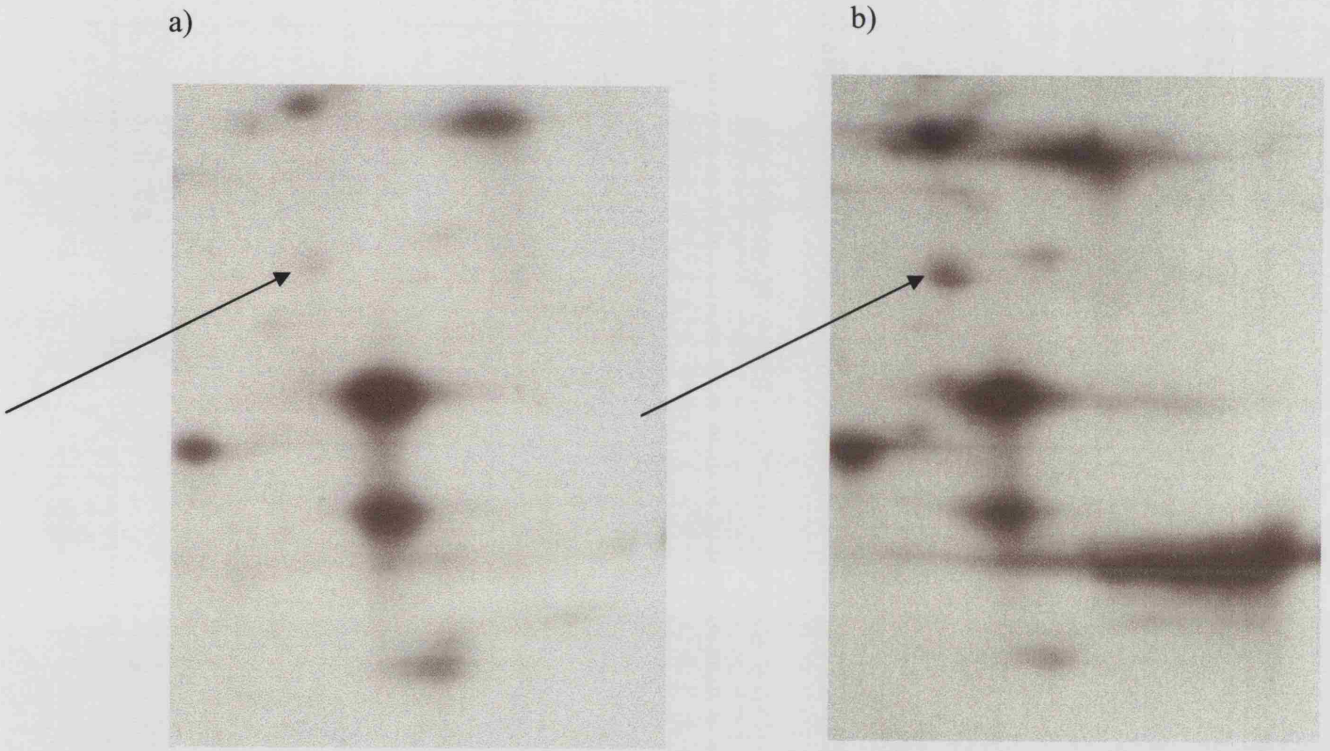


Figure 4.10 a) and b)

Enhanced gel image comparison of a) control and b) sorbic acid gels with the arrow identifying the position of spot number 301 on the sorbic acid 1 hour 15 µg gel and its comparative position of the control gel.

This sorbic acid stressed protein having been switched on compared to the control.

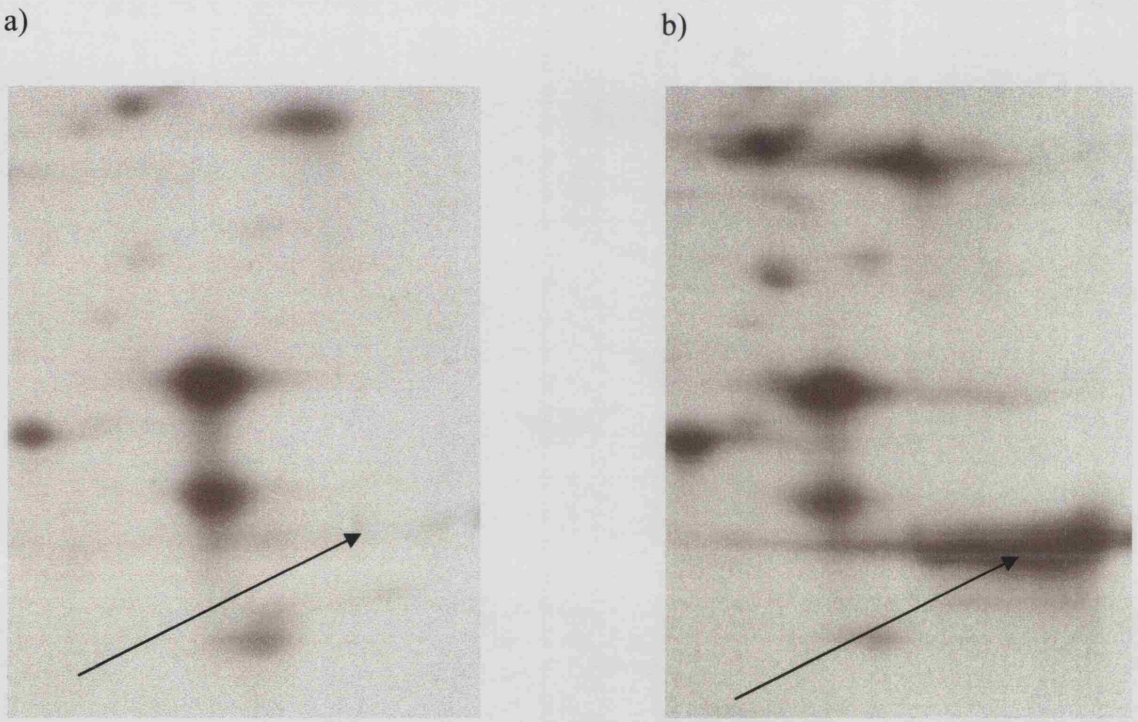


Figure 4.11 a) and b)

Enhanced gel image comparison of a) control and b) sorbic acid gels with the arrow identifying the position of spot number 315 on the sorbic acid 1 hour 15 µg gel and its comparative position of the control gel.

This sorbic acid stressed protein having been switched on compared to the control.

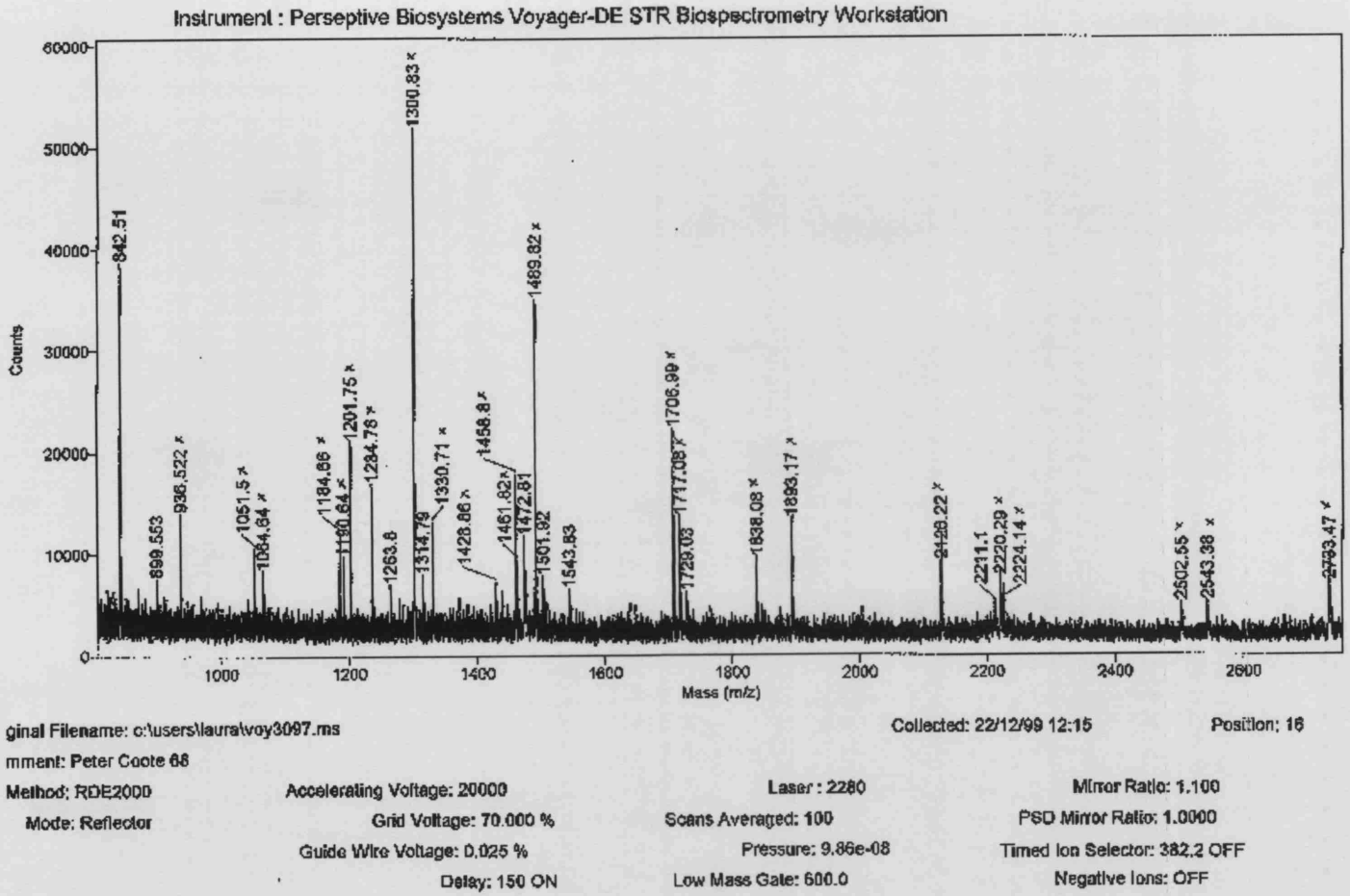


Figure 4.12

A scanned image from the mass spectrometry analysis of spot number 68, after the peptide digest.

4.3. Discussion

This Chapter describes the use of the proteomic techniques of 2D-PAGE, using both traditional staining and pulse labelling with ^{35}S methionine to identify cytosolic proteins with altered expression due the to exposure to sorbic acid. Two different sets of 2D-PAGE gels, one with the same amount of protein loaded per gel (15 µg) and the other with the same amount of radio-label per gel (300,000 CPM) were used to conduct the comparison across each control and sorbic acid pair for 1 and 3 hour time points. The gel sets were further cross-matched to ensure that any differences detected were real changes in expression and not artefacts, thus giving further confidence to the results. By studying samples taken after a short time frame after sorbate addition, the proteins identified would be those that having undergone an immediate change in expression.

The short-listed proteins that were found to have a changed level if expression in response to the weak acid stress were to be investigated further via phenotypic deletion mutant analysis to identify any that could be possible proteins involved in stress resistance. The findings were further compared to other later studies published by other groups using both proteomic and genomic approaches to identify elements involved in the stress response to sorbic acid in yeast.

4.3.1. Successes and issues with the proteomic approach used in this study

As an indication of success of the separation technique, the total number of proteins separated and detected were counted for each gel and ranged from 700 to 1000 proteins. This is probably the best that can be expected as the most that can be usually separated by this technique is a maximum of 1,200 (Navarre *et al.*, 1995). The techniques used in this study were aimed to extract only the cytosolic proteins and did not attempt to extract or solubilise the membrane bound protein fraction.

The clarity and quality of the separation was concluded to be of a good standard when compared to other published studies (Boucherie *et al.*, 1995, Blomberg, 1997 both in yeast and Rabilloud *et al.*, 2001). Furthermore, the numerous repeats conducted over a number of data sets ensured that if any of the proteins of interest

were of low abundance (discussed in depth by Navarre *et al.*, 1995), a correlation across the whole set gave confidence that the find was real and not an artefact.

Often due to two spots having the same pI and MW or from issues with resolution an excised spot when analysed can be comprised of two proteins on top of each other. Alternately, the peptide fingerprint identified by mass spectrometry could be aligned to two different identities in the MASCOT database. The MOWSE score is thus useful to give an indication of the level of 'fit'. On further study of each of the MS fingerprint traces for each of the excised spot in question, the trace showed that the peptides of two proteins had been excised from the gel and thus were present. Both of these proteins having similar molecular weights and pI values and so both identified proteins were assessed as candidates involved in the stress response.

At the time that the proteomic study was conducted the techniques used gave the highest throughput of protein identifications possible, but this was limited by the lack of initially good data analysis software and where used was only semi-automated. In just a few years with the rapid development of new technologies it is now possible to rapidly screen the whole proteome and genome and perform an analysis of the changes across the whole cell due to the stress. These studies provide a far larger data bank of information to use and thus were compared to give confidence to these study results.

4.3.2. The proteins identified as of interest from the proteomic analysis

This study performed a study of the effect of the sorbic acid stress using exponential growing cells which were harvested 1 and 3 hours after addition, thus detecting any immediate changes in expression. The results of the matches between the control and the sorbic acid stress both over 1 and 3 hours identified not only the proteins that remained unchanged, but also those that were induced by the stress and remained switched on, those that were switched on immediately but then were tuned off after 3 hours and those that were induced later. A couple of proteins detected were of this late onset nature but their overall increase in expression was relatively small compared to the majority of other proteins that were induced as a result of the stress and remained switched on.

The proteins identified by this study were involved in the immediate stress response to sorbic acid. Other studies such as de Nobel *et al.* (2001), also used a similar proteomic approach to study the effect of sorbic acid on yeast, but the cells were harvested after reaching late exponential phase and thus this study differed in that the identified proteins were involved in the adaptation to the stress rather than the immediate effect.

A post comparative analysis has also been conducted between the findings of this study to studies published later using other genomic and proteomic techniques (Schuller *et al.*, 2004, Mollapour *et al.*, 2004 and de Nobel *et al.*, 2001). The differences in the various techniques used were noted, as these could offer variability and differences in result patterns seen. A summary of these findings can be found in Table 4 .4 but are discussed fully below.

4.3.2.1. Ach1p - Acetyl CoA Hydrolase

The protein Ach1p or Acetyl-CoA hydrolase was identified from this study as a protein being induced as a result of the sorbic acid stress and observations showed that this protein was induced and remained so in both time points. The protein when analysed and compared to the MASCOT database scored a value of 67, denoting a good match to its putative identity. However, spot number 48 when excised from the gel was shown to be comprised of two proteins, thus the fingerprint providing two protein identities, Ach1p and Ero1p. The match between the experimental and published pI and MW values for Ach1p correlated well providing further confidence to the protein identity with the spot.

The protein Acetyl-CoA hydrolase is reported to be involved in lipid, fatty acid and sterol metabolism and catalyses the hydrolysis of the acyl CoA chains, releasing CoA and acetate. A study conducted by Moskvina *et al.* (1998) showed that many genes have stress response elements (STRE's) upstream of their promoter and transcription driven by STRE's was demonstrated to be induced by a variety of stress conditions, such as heat, osmolarity, oxidative or starvation. This study in particular showed that the gene *ACH1* had four putative stress response elements in the promoter, providing

evidence that such a gene maybe key in the general stress response of *S. cerevisiae*, enabling yeast to activate a protective response against a broad variety of stresses.

Further evidence to the involvement of this protein in the stress response to sorbate has also been reported in other studies (Schuller *et al.*, 2004 and de Nobel *et al.*, 2001). The work of de Nobel *et al.* (2001) used a similar proteomic technique as this study and showed that this protein was also upregulated allowing the cell to adapt to the sorbic acid stress. The studies by Schuller *et al.* (2004) using DNA microarray techniques also identify this protein to be induced as a result of the sorbic acid stress. In this study they concluded that the gene *ACH1* was dependant upon the transcription regulators Msn2p and Msn4p for its induction to produce the eventual protein Ach1p. Furthermore, Moskvina *et al.* (1998) reported the possibility of these two transcription factors bound specifically to the STRE's, and when a cell was deficient in these factors it was hypersensitive to severe stress conditions.

There are many genes involved with metabolism in the yeast that are either switched on or further induced in order to cope with the stress. Clearly *ACH1* is one of many involved in the metabolic pathways that are required to be stimulated further for the cell to cope with the stress. Thus the exact role of Ach1p involvement clearly needs further study and thus is a clear candidate for further investigation. The early evidence that the gene coding for Ach1p contained possible stress response elements within the upstream region also provided further evidence to select this protein for phenotypic analysis. However, the studies of Schuller *et al.* (2004) and de Nobel *et al.* (2001) gave further confidence towards this selection.

4.3.2.2. Ero1p – catalysis of disulphide bond formation

The second protein excised from the same spot number 48 as Ach1p was Ero1p, and similarly, this protein being induced as a result of the sorbic acid stress and observations showed that this protein was induced and remained so in both time points. The Mowse score from the database, however, was more border-line as to a significant match being sought with an identification value of 53. Furthermore, the correlation between the experimental and published pI and MW values (especially for the pI) for this protein was not so high, causing a level of uncertainty with this identification.

Other studies of de Nobel *et al.* (2001), Schuller *et al.* (2004) and Mollapour *et al.* (2004) were also consulted to determine if any of these identified Ero1p involvement in the sorbate stress response. None of the studies quoted Ero1p being either induced or repressed as a result to sorbic acid, thus shedding further doubt to Ero1p involvement in this stress response. However, further literature was consulted to the role Ero1p could play in any possible stress response.

Ero1p is reported to be involved in disulphide bond formation in the endoplasmic reticulum (E.R.) and is thought to help maintain the correct redox state in the E.R. The structure of many proteins entering the secretory pathway are dependant upon being stabilised by disulphide bonds and thus to support this activity, the E.R. must maintain a strongly oxidising environment contrary to the cytosol (Pollard *et al.*, 1998). Studies by Pollard *et al.* (1998) have shown that there is a specific cellular redox complex required for disulphide-linked protein folding in the E.R. and that Ero1p is an essential component of this. Loss in *ERO1* function results in the accumulation of unfolded proteins within the E.R. Furthermore, a stress response often results in malformed proteins thus this protein could be important in the protection of such events during stress.
Thus it could be possible that this protein maybe involved in the recovery of a cell from a stress, however, all other evidence seems to conclude that it is not the case for this protein and thus this protein is a low priority candidate for further analysis.

4.3.2.3. Cys4p – Cystathione beta-synthase

Cys4p or Cystathione beta-synthase was the only spot to be identified by mass spectrometry to spot number 68, and scored a highly significant Mowse score of 302. The match between the experimental and published pI and MW values for Cys4p was close, providing further confidence to the protein identity with the spot. From the observations from the gels this protein was induced 6 fold over the control in the sorbic acid stressed samples for both 1 and 3 hour time points.

To give further confidence to the involvement of Cys4p in the sorbic acid stress response, other studies were compared. The phenotypic screen of diploid *S. cerevisiae* mutants in the presence of sorbic acid conducted by Mollapour *et al.* (2004) reported the loss of the *CYS4* gene resulted in the mutant strain being sensitive to the presence of sorbic acid.

Cystathione beta-synthase is located within the cytoplasm and is the first enzyme within the cysteine biosynthesis pathway converting serine and homocysteine to cystathione. Studies conducted by Oluwatosin & Kane, (1997) have shown that the *cys4* mutants have an inactive vacuolar ATPase resulting in a less reducing cytosol due to the loss of this gene and also display pH-dependant growth phenotypes. Supplementation into the media of this amino acid cysteine can not relieve this loss in activity and in addition, cysteine is required for glutathione synthesis, and thus a *cys4* mutant will be unable to synthesise both cysteine and glutathione. Glutathione has numerous functions including metabolism, transport and possibly antioxidant protection for the cell and plays an essential role in cell growth (Oluwatosin & Kane, 1997). Mollapour *et al.* (2004) also showed that other genes that were part of the glutathione biosynthesis pathway resulted in sorbic acid sensitivity, concluding that such antioxidant activities would provide resistance to the cell by protecting a specific critical target and showing the importance of maintaining a reducing environment in sorbic acid stressed cells.

Due to such a high MOWSE score, good correlation with published MW and pI data, possible involvement in cell protection this protein was undoubtedly of interest and a candidate for further study.

4.3.2.4. Atp2p – ATP synthase

The protein Atp2p or ATP synthase (beta chain sub-unit) was identified along with another protein Hsp26p from the spot 86 as being induced as a result of the sorbic acid stress. The observations from the gels showed that both proteins were induced 9 fold comparing the 1hour sample to the 3 hours, thus the induction of this protein gradually increasing with time. The protein Atp2p when analysed and compared to the MASCOT database scored the higher value of the two proteins 92, relating to a significant score and thus a good match to its identity. The correlation the experimental and published pI and MW values for Atp2p were also close, providing further confidence to the protein identity. The studies of de Nobel *et al.* (2001) also reported the induction of ATP2p as a result of sorbic acid stress using similar proteomic techniques, thus providing further evidence of this protein's involvement in the sorbate stress response in yeast.

ATP synthase (beta chain sub-unit) makes up part of the mitochondrial ATP synthesis module, catalysing ADP to ATP and has been reported to be involved in the stress responses in particular being up-regulated due to sorbic acid (de Nobel *et al.*, 2001). Other researchers have also discussed the need for this protein (Holyoak *et al.*, 1996 and Piper *et al.*, 1997) as being critical element for the upregulation of energy generation during adaptation to sorbic acid stress and its involvement is supported by results (Holyoak *et al.*, 1996) showing excess consumption of ATP as a result of sorbic acid stress.

This protein as part of the first short-list due to the extent of study already conducted on this protein and its known involvement in the stress response extensively studied.

4.3.2.5. Hsp26p – Heat shock protein (26kda)

Hsp26p or Heat Shock protein (26 kda subunit) as already discussed was the second of proteins to be identified to spot 86, however, with a MOWSE score of 51 this was only just a significant match. Also as discussed before this protein was induced 9 fold comparing the 1 hour sample to the 3 hours, thus gradually increasing with time.

When comparing the experimental and published pI and MW values for Hsp26p there was a difference in the MW values, the experimental reported in this study to be 49,000 where as the published 25,000 (a clear difference). This difference could be due to post-translational modifications or protein aggregation, however, this does give rise to doubt these findings.

When comparing the results of other studies, many of these report finding wither Hsp26p to be induced or the *HSP26* gene deletion mutant more sensitive in the presence of sorbic acid (de Nobel *et al.*, 2001 and Schuller e*t al.*, 2004) using both proteomic and DNA microarray approaches. This protein has been reported already (de Nobel *et al.*, 2001) to be synthesised in response to cellular exposure to various stresses such as temperature, but has also been shown to be induced by other types of stress such as free radical damage or weak acids e.g. sorbic acid (de Nobel *et al.*, 2001). In addition, Moskvina *et al.* (1998) identified Stress Response Elements (STRE's) as part of the promoter in *HSP26*, again linking this gene and protein to a stress response function and therefore of interest for further study. Studies by Blomberg (1997) investigating osmoresponses in yeast revealed that *HSP26* was shown to be osmo-induced as a result an osmotic shock by salt or sucrose.

Despite the results from this study showing a less confident match with this spot, the evidence in other published data leads to the conclusion that Hsp26p is most definitely involved in the stress response within in yeast and would possibly be taken for further investigation.

4.3.2.6. Ilv5p – Ketol-acid reductoisomerase

As for the previous spot, two proteins again were excises and identified to be part of spot number 144. The first protein Ilv5p or Ketol-acid reductoisomerase scored a Mowse score of 129, thus a significant match to this identification. The observations of the gels demonstrated that this protein was induced over 21 times comparing the control to sorbic acid stresses samples for both 1 and 3 hours. However, when comparing the experimental and published MW and pI data, these showed poor correlation, especially for the pI (6.8 compared to the published 9.8).

Other studies also report finding the gene *ILV5* being involved in the stress response (Schuller *et al.,* 2004), however, this gene is reported as being repressed rather than as in this study being induced. Other research by Blomberg (1997) also reports the repression of Ilv5p as a result of saline stress on the yeast cell and so again providing conflicting evidence to this studies finding.

Therefore, on the basis of inconclusive results towards the actual identity of the excised protein to Ilv5p but also further conflicting published data that this protein is actually repressed in response to stress rather than induced, leads to the decision not to choose this study for further analysis.

4.3.2.7. Adh1p – Alcohol dehydrogenase

A second protein also matched to spot 144 was the protein Adh1p, and the identification was matched to this protein with a significant score of 83. The experimental and published MW and pI values both correlated well, and the gels indicated that this protein was induced in both 1 and 3 hour time points as a result of the stress. Schuller *et al.* (2004) also identified that a deletion of the gene *ADH1* resulted in sensitivity of the strain to sorbic acid.

The protein Adh1p, an alcohol dehydrogenase enzyme which catalyses the reaction of alcohol to an aldehyde or ketone and NADH and belongs to a major group of proteins involved with energy metabolism (YPD database: www.proteome.com). Many of these genes categorised as general maintenance or energy metabolism have been extensively investigated and thus this protein was not chosen for further investigation.

4.3.2.8. Tdh2p and Tdh3p– glyceraldehydes 3-phosphate dehydrogenase subunits

Both Tdh2p and Tdh3p - glyceraldehyde 3-phosphate dehydrogenase subunits (two parts of a three piece protein complex) were both identified from two different excised proteins as being up-regulated as a result of the sorbate stress. Tdh2p scored a MOWSE value of 107 and Tdh3p a score of 58, the first score more significant and thus better match than the latter. This was also reflected in the comparison of experimental and published pI data. Tdh2p compared well to both data sets, where as the correlation for the MW for Tdh3p was not so good (20,000 compared to 36,000). Both proteins were found to be switch on as a result of the sorbic acid stressed when compared to the untreated cells.

The gene *TDH2* was also identified by de Nobel *et al.* (2001) using DNA microarray techniques as being induced as a result of the sorbate. Furthermore, this study also detected the other part of the subunit Tdh1p also having been induced. Mollapour *et al.* (2004) using phenotypic analysis of gene deletion mutants, found that loss of the gene *TDH3* resulted in increased sensitivity to sorbate. However, findings of Blomberg (1997) using a proteomic approach researching yeast responses to saline stress, found that the expression of Tdh3p was actually decreased, lowering consumption of glerceraldehyde-3-phosphate., thus giving conflicting results for this protein

Tdh3p, on the basis of conflicting published evidence for Tdh3p involvement in the stress responses but also due to poor confidence in the data matches was not chosen for further analysis. However, it was decided that Tdh2p would also not be chosen to being classed as a general maintenance proteins involved in the glycolysis pathway.

4.3.2.9. Hcr1p – Hypothetical protein

The hypothetical protein Hcr1p (YLR192C) was identified to match spot number 187 with a score of 84. Both the experimental and published data for the MW and pI correlated well, and this protein was shown to be induced for both time points as a result of the sorbate stress.

Comparing the results of others studies, none identified this hypothetical protein as showing any sensitivity or resistance to the sorbate stress. However, the literature indicated that this protein could be a component of a translation initiation factor eIF3 and had a high similarity to the human eIF3 p35 subunit. Block *et al.* (1998) showed that the human eIF3 was part of a large multi-subunit complex that played a central role in the initiation of translation. Further studies conducted by Valasek *et al.* (1999) demonstrated a similar role for the protein in *S. cerevisiae*. Thus the combination of a high Mowse score, good comparison of experimental MW and pI to those in the literature, no known function to this protein in yeast but with putative reported links to a translation initiation factor, this protein was selected for further investigation. Even though other later studies have not shown its presence this would still not have prevented its selection for further analysis.

4.3.2.10. Cph1p – cycophilin (peptidyl propyl cis-trans isomerase)

The protein Cph1p or cycophilin (peptidyl propyl cis-trans isomerase) was identified to match the spot 315 with a significant score of 122. The experimental and published MW and pI data correlated very well, and from the gel the protein was switched on after 1 hour but had disappeared after 3 hours. The work of de Nobel *et al.* (2001) also identified this protein using DNA microarray, as being induced as a result of sorbate stress, thus providing further evidence and confidence in this match.

Cycophilin (peptidyl propyl cis-trans isomerase) has been shown to be present in the cytosol and to be induced by both high salt and sorbic acid. It is reported to have two stress response elements present within the promoter and thus being linked to various stress responses within the cell (Moskvina *et al.*, 1998). This finding also relates to the studies of de Nobel *et al.* (2001) who also conclude that the *CPH1* gene was up-regulated in response to the stress by the binding of the stress inducible transcription factors Msn2p and Msn4p. Studies by Dolinski *et al.* (1997) have shown that cph1p is highly conserved and can be induced by heat shock, thus is thought to accelerate the folding of other proteins, however, they go onto conclude that the protein is not essential for viability.

The protein Cph1p was chosen for further investigation, as like Ach1p described earlier has clear links to the stress response and the study has provided good evidence for its identity.

4.3.3. Summary of proteins chosen for investigation by phenotypic deletion analysis

The proteins that have been chosen for phenotypic deletion mutant analysis are those that have reported functions within stress responses and have a highly significant match from the fingerprint to protein identity from the MASCOT database. The proteins that were identified as general maintenance proteins, for example, those that are involved with glycolysis and other general biochemical pathways, such as, glyceraldehyde 3-phoshate dehydrogenase were not chosen for further study due to already extensive studies completed on them. Even though some of these general maintenance proteins were shown to have involvement in the stress response, and indeed published results demonstrate this to the case these were not chosen for the first round of analysis. It was hoped if time permitted these proteins involved in energy metabolism and general maintenance could be studied further to reveal more interesting results.

Other proteins were not chosen due to poor correlation between the experimental and published data raising doubt of the identity such as for Ero1p. Other proteins such Ilv5p clearly showed conflicting results in the induction in this study verses repression in other studies, along with poor pI correlation, concluded that this identity for this protein was incorrect.

The proteins that were chosen for further study were Ach1p (Acetyl-CoA hydrolase - spot 48), Cys4p (Cystathione beta-synthase - spot 68), the hypothetical protein Hcr1p (YLR192C - spot 187) and Cph1p (cycophilin peptidyl propyl cis-trans isomerase – spot 315). In addition the protein Hsp26p (heat shock protein 26 - spot 86) which was less well matched but had overwhelming evidence of its involvement in the stress response was also chosen.

Of those proteins excised and analysed by peptide mass fingerprinting, about half were shown to be of interest for further study. The combination of the Mowse score, experimental verses reported molecular weight and pI co-ordinates and using information regarding the proteins possible function within the stress response enabled a further short listing of proteins. However, by comparing published to experimental data and analysing their possible functions from the literature a list of candidate proteins were identified. It is clear from the diversity of proteins within the list that as a result of a stress from sorbate there is no one single cellular response, but a great complexity of different systems protecting the cell.

Chapter 5 – Analysis of hypersensitivity to weak acid preservatives in selected yeast deletion mutants

5.1. Introduction

S. cerevisiae has long been the eukaryotic model of choice for many different types of studies on cellular physiology. Although yeasts have greater genetic complexity than bacteria, they were one of the first organisms to be fully sequenced and analysed at the genome level (Brul *et al.*, 2002), thus sharing many of the technical advantages that have permitted rapid progress in molecular genetics. Some of the properties that make yeast particularly suitable include the ease of replica plating and mutant isolation, as well as a defined genetic system and importantly a highly versatile DNA transformation system (Sherman, 1991).

To indicate the need for such an organism, research has shown (Winzeler *et al.*, 1999) that up to 30% of the genes implicated in human disease were shown to have yeast homologs. Therefore, determining the function of these yeast homologs could provide insight into disease mechanisms within humans. Furthermore, other genes essential for yeast viability, but which lack a human homolog, might be new possible targets for antifungal drugs (Winzeler *et al.*, 1999). The event of such advances has also enabled for this organism to be used in the analysis of effects of different preservation conditions on yeast cellular physiology (Brul *et al.*, 2002), and is of major relevance in this study investigating weak organic acid sensitivity in yeast.

5.1.1. *S. cerevisiae* genome deletion libraries

The EUROFAN project was set up by the European Yeast Genome Sequencing Network to establish the biological function of novel genes revealed by the *S. cerevisiae* genome sequence. Their aims were to accelerate the progress of research by creating a genetic archive and stock centre (called EUROSCARF) comprising of a collection of yeast strains containing specific deletion mutations and plasmids for particular genes produced in the FY parental background, using in particular the parental strain FY1679-28c (EUROFAN Deletion Project and EUROSCARF Collection Websites). Thus their goal was to generate a complete set

of yeast deletion mutants in order to assign function to the unknown ORF's through phenotypic analysis of the mutants. In 2004, 8 years after the completion of the genome sequence, 20-25% of the ORF's still had no known function (Winzeler *et al.,* 1999 and http://www.yeastgenome.org).

However, at the time of the studies discussed within this Chapter, mutants deleted in genes for the yeast proteins identified in Chapter 4 were unfortunately not available from the EUROSCARF collection. Thus a different source was sought, namely from the *Saccharomyces* Genome Deletion Project (a collaboration of different American and European laboratories) via Research Genetics, who with a similar research goal had produced a far larger collection. Within this collection all of the deletion mutants were available in a BY parental background (as with strain FY, S288C-derived). All of the mutants contain a *kanMX4* cassette replacing the single ORF deletion from the yeast genome, this cassette being tagged with one or two unique 20mer sequences (a "barcode"), enabling detection. Four mutant collections were generated by the project, haploid disruptants of both mating types, homozygous disruptant diploids (for non-essential genes) and heterozygous disruptant diploids (essential genes only) (Winzeler *et al.,* 1999). For this study, the haploid "a" mating type collection of deletant strains was used.

5.1.2. Phenotypic analysis techniques to reveal gene functions in *S. cerevisiae*

As discussed previously, the completion of the analysis of the *Saccharomyces cerevisiae* genome sequence has identified well over 6000 predicted genes (Blomberg, 1997) but completing the genomic sequence of an organism is not sufficient to elucidate biological function. Thus the scientific community has now shifted its' focus to not only identifying the structure and assigning function to these new genes (Lockhart & Winzeler, 2000) but also investigating interactions and organisation of genetic pathways that control the production of proteins as well the roles they in play in physiology of an organism (Navarre *et al.*, 2002 and Dhand, 2000).

Within a cell it is clear that a multitude if metabolic and regulatory pathways are required for both the operation in 'normal' conditions but also in a more hostile

environment to survive (Pandey & Mann, 2000). The work by other researchers (de Nobel *et al.*, 2001, Mollapour *et al.*, 2004 and Schuller *et al.*, 2004) have shown using genomic and proteomic techniques just how many different systems are induced and repressed by sorbic acid stress, these patterns of expression changing in different environments, indicating the sheer complexity of studying such systems.

Phenotypic analysis of mutant strains with the genes of interest deleted from the genome, i.e. deletion mutant strains, can be studied using particular stress condition to determine their function. By looking for reductions in growth within media containing such stress conditions e.g. sorbic acid, easily identifies mutants that are sensitive compared to an unaffected parent control strain. Thus proteins or genes that have been identified in previous genomic or proteomic studies that have changed expression in response to the stress, such as weak acid in this case, can be further analysed and confirmed having possible functions in sorbate resistance.

5.1.3. Weak acid hypersensitivity in tryptophan biosynthesis mutants

As a result of studies in this Chapter, phenotypic analysis identified differences in weak acid sensitivity displayed by two genetically-similar parent strains FY1679-28c and BY4741. The FY strain was that used in the proteomics study to identify key proteins with changes in expression due to sorbate, the BY strain is the parent used in the phenotypic analysis in this Chapter. These differed in that the FY parent is a tryptophan auxotroph (*trp*-), whereas the BY parent a tryptophan phototroph (TRP^+).

Plants and microbes (including yeast) can biosynthesise tryptophan from precursors through a pathway consisting of five steps starting with chorismate and catalysed by the products of *S. cerevisiae* genes *TRP1-5* (Hutter & Niederberger, 1986). Using strains with both increases and decreases in each of the five tryptophan biosynthesis enzymes, Niederberger *et al.* (1992) were able to show that no one step was actually rate limiting. However, when all five were simultaneously increased, a substantial elevation in the flux to the production of tryptophan was observed. Within the studies in this Chapter, tryptophan biosynthetic mutants are used to investigate the effects of amino acid auxotrophy has upon cellular hypersensitivity to weak acids.

Observations by Mollapour *et al.* (2004) and Schuller *et al.* (2004) both identified using phenotypic analysis of yeast deletions mutants (in haploid and diploid backgrounds) in the presence of sorbate, deletion of a number if amino acids genes (including all from the tryptophan biosynthesis pathway) incurred sorbate sensitivity. These results confirming further the significance of such genes as a target of this study.

Furthermore, this study sorts to confirm the findings of Piper *et al.* (1998) that the *pdr12* mutant was still sorbate sensitive if in a TRP^{+} background.

5.1.4. The multiple inhibitory actions of weak acids in yeast

The increasingly lower amounts of preservative being permitted for use and the resistance to these preservatives, has become a huge issue for the food and beverage industries (Booth & Kroll, 1989). The resistance of yeast to the small number of weak organic acids that are permitted for food preservation allows these organisms to cause large-scale spoilage of preserved foods and beverages (Piper *et al.*, 2001). As extensively discussed in Chapter 1, resistance to weak acid stresses must involve overcoming intracellular pH reduction, anion accumulation in the cell and the disruptive effects of the acid on membrane transport and/or permeability (Piper *et al.*, 2001).

Many different intracellular targets of weak acid stress proposed in the literature are discussed in Chapter 1, however, no single mechanism thus so far proposed can fully explain the action of sorbic acid and acetic acid under all preservation conditions. As has been shown in a number of studies including this one and Mollapour *et al.* (2004) and Schuller *et al.* (2004) the results of a stress event changes the expression of hundreds of different genes and thus proteins in response to coping with the stress and thus it can be expected that there is undoubtedly more than one target involved and thus growth inhibition results from a combination of cellular inhibitions.

5.1.4.1. Differences in the yeast stress responses to the weak acids; acetate and sorbate

Though both acetic and sorbic acid are weak organic acid food preservatives, many of the determinants of acetate resistance in *S. cerevisiae* differ from the determinants of resistance to the more lipophilic sorbate and benzoate (Chapter 1 and Piper *et al.*, 2001) and it has been shown that, though acetic and sorbic acids have similar pKa values, they have very different antimicrobial activities (Salmond *et al.*, 1984). Acetate could possibly be solely mainly to disrupt the intracellular pH (as in Figure 1.1), whereas the more hydrophobic sorbic acid does not generate a drop in pH_i but is probably mainly inhibiting cells through the disordering of the membrane structure, (Bracey *et al.*, 1998). Studies carried out by Stratford & Anslow (1998) have shown that a strong inhibitory action via the membrane increases as the weak acids become more lipophilic.

Piper *et al*, (1998) found contradictory results when studying acetate resistance of two mutant strains Δ*pdr12* and Δ*azr1*. Thus the group constructed a double Δ*pdr12* Δ*azr1* mutant to determine which of the proteins, Pdr12p or Azr1p, were more important for acetate resistance (Bauer *et al.*, 2003). Thus research conducted within this study and in that of Bauer *et al.* (2003) sort to investigate further these differences for both sorbate and acetate.

5.1.5. A model for yeast resistance to weak acids

Holyoak *et al.* (1996) showed that plasma membrane H^+-ATPase plays a critical and energy-demanding role in adaptation of yeast to weak acid stress and sufficient ATP is required to drive this process without depleting cellular ATP levels needed for growth. It is known that the H^+-ATPase consumes much of the ATP generated by the cell as such maximal activity is rare and energetically expensive to maintain (Serrano, 1991). Thus high activity of this membrane protein over long periods of time might be unsustainable (Piper *et al.*, 1997). A weak acid inducible stress protein Hsp30 plays a role in this adaptation by partially down-regulating the H^+-ATPase sorbate induced activity, limiting excessive energy consumption and thus conserving levels of ATP during extended exposure to weak acid (Braley & Piper, 1997).

Further studies by Piper *et al.* (1998) and Kren *et al.* (2003) have shown that for weak acid adaptation, the strong War1p transcription factor-dependent induction of the ATP-binding cassette (ABC) transporter, Pdr12p occurs, i.e. the system for energy dependant efflux of weak organic carboxylate anions. It is proposed that Pdr12p benefits the cell in two ways, by lowering intracellular acid levels and balancing the charge movement, aiding a higher level of catalysed proton extrusion (see Figure 1.2). The Pdr12p induction in sorbate-stressed cells is so strong that levels of this transporter in the plasma membrane approach those of the most abundant plasma membrane protein, plasma membrane H^+-ATPase (Piper *et al.*, 1998 and 2001). Furthermore, the inhibitory effects of different organic acids on cells of the *pdr12*Δ mutant indicate that Pdr12p confers resistance to sorbate, benzoate and aliphatic short chain (C3-8) carboxylic acids of reasonable water solubility (Holyoak *et al.*, 1999 and Hatzixanthis *et al.*, 2003). The discovery of War1p and the lack of involvement of any other known stress regulators for this response shows that the weak acid response must be quite distinct from other characterised stress responses in yeast (Kren *et al.*, 2003).

Tenreiro *et al.* (2000) analysed the *S. cerevisiae AZR* gene, encoding an integral trans-membrane protein of the major facilitator superfamily (MFS) class of transporters which are required for multiple dug resistance. This was shown to be required for yeast adaptation to high stress imposed by acetic acid and also for resistance to azoles, from the susceptibility of *azr1*Δ deletion mutants in the FY background to these compounds. *AZR1* gene expression also reduced the duration of acetic acid induced latency, though Azr1p was not implicated in yeast adaptation to acetic acid by catalysing the active efflux of acetate (Tenreiro *et al.*, 2000). Clearly each of these elements has a role in weak acid resistance, and this study seeks to confirm that they are involved and identify possible roles within the complex stress of the cell to weak acids.

5.2. Results

5.2.1. Phenotypic analysis of growth inhibition of yeast deletion mutants in the presence of sorbate using the Bioscreen turbidometer

Increasing concentrations of sorbic acid (0 to 1.8 mM) in YPD medium at pH 4.5 were added to growing cultures of *S. cerevisiae* strains, BY4741 (parental strain) and deletion mutants *ach1, hsp26, cph1, cys4* and *hcr1* (hypothetical gene YLR192C) identified from the proteomic study in Chapter 4. The changes in growth for each of the strains were measured simultaneously in the presence of varying levels of sorbate stress using the Bioscreen turbidometer and the results were analysed from three sets of data.

Figure 5.1 depicts an example graph showing the resultant logarithmic optical densities (600nm) measured against time (hours) for the BY4741 parent growing in the presence of sorbate (0, 0.45, 0.9 and 1.8 mM), with error bars. The graph shows as the sorbic acid concentration is increased so the resultant growth rates gradually reduce, furthermore, the yields for only the highest sorbic acid concentration are reduced for the BY4741 strain. This pattern of results was repeated for all the other mutant strains when plotted in the same way (data not shown).

Figure 5.2 shows the effect on the growth rate (h^{-1}) with increasing sorbate concentration for all the *S. cerevisiae* parent and mutant strains tested. The graph shows for all of the strains as the sorbic acid concentration increases so the growth rates for each are reduced. However, there is very little difference between the growth rate plots between any of the strains in response to the sorbic acid stress. Only one strain showed any differences in growth rate, this being for the hypothetical deletion mutant *hcr1*. Even without any sorbic acid added the growth rate was slower for this strain, however, on adding sorbic acid the growth rates for *hcr1* were not much less that for the other strains screened.

5.2.2. Analysis of growth inhibition of the yeast knock out deletion mutants in the presence of sorbate using zonal inhibition agar plates

The growth of the deletion knock out mutant strains was compared to the BY parent strain BY4741, but also the additional diploid parental strain BY4743, using zonal inhibition YPD agar plates containing increasing levels of sorbic acid, at concentrations of 0, 0.45, 7.2. 14.4 and 50 mM.

Figures 5.3 a), b) and c) show photographs of the resultant growth after 24 hours incubation at 30°C depicting only 0, 14.4 and 50 mM sorbate concentrations. The comparison of each of the strains at 0 mM against the growth at 14.4 and 50 mM sorbate showed that there was no sign of inhibition for any of the strains on the 14.4 mM plate. Only on the 50 mM plate was there any sign of inhibition and that was for the *cys4* strain.

5.2.3. Analysis of growth inhibition of two parental yeasts strains (FY and BY) in the presence of sorbate using the Bioscreen turbidometer

An observation from a previous bioscreen experiment (data not shown) noted that the FY parental strain was far more sensitive to sorbate than any of the BY strains. Thus a direct comparison of the level of growth inhibition for the two different *S. cerevisiae* parental strains was conducted, the FY1679-28c (*MAT*a and *trp*-) strain from the proteomic study (Chapter 4) and the BY4741 (*MAT*a and TRP^+) strain used for the phenotypic evaluations within this Chapter. Increasing concentrations of sorbate (0, 0.45, 0.9 and 1.8 mM) were added to growing cultures of the parental strains in YPD medium at pH 4.5 and changes in growth were measured using the Bioscreen turbidometer.

The resultant logarithmic optical densities (600nm) over time (hours) were plotted for the strains with increasing sorbate and the resultant growth rates calculated (data and graphs not shown). Figure 5.4 depicts the changes in the growth rates (h^{-1}) for both parent strains over the range of sorbic acid concentrations (0 to 1.8 mM). From the graph, the FY1679-28c strain had a far slower growth rate even in the absence of

any weak acid compared to that the BY parent. On the addition of the weak acid both strains were inhibited the FY (*trp*-) strain more so than the BY (TRP^+) strain.

5.2.4. Bioscreen analysis of the growth inhibition of five tryptophan biosynthesis pathway mutants compared to that of the BY parent as a result of sorbate stress

To investigate the effect of tryptophan auxotrophy further in the presence of weak acids, the growth of five tryptophan biosynthetic pathway mutants were compared to the BY parent strain (BY4741). These were cultured in the presence of increasing concentrations of sorbic acid (0, 0.45, 0.9, 1.8 and 3.6 mM) in YPD medium at pH 4.5. A comparison of the growth inhibition for each of the strains was conducted and changes in growth were measured using the Bioscreen turbidometer.

Figures 5.5 a) and b) show a comparison of the results for only a) BY4741 and b) *trp5*Δ plotting logarithmic optical density (600nm) against time (hours). The graphs represent the analysis of three data sets, with the plots also showing calculated standard deviations for the data. The resultant growth plots for each of the strains over a range of sorbate concentrations show that the *trp5*Δ mutant is clearly more sensitive than the wild type to the inhibitory effects of the stress especially at the higher concentrations, under the culture conditions. However, this strain does seem to generally less robust than the parental strain. The *trp1*Δ, *trp2*Δ, *trp3*Δ, and *trp4*Δ, mutants were also compromised under these conditions (results not shown) their growth being essentially similar to that of the *trp5*Δ mutant cells. Furthermore, when comparing all of these strains to the BY parent they were all generally less robust than the parent.

5.2.5. The effect of tryptophan supplementation to the media on the growth of five tryptophan biosynthesis pathway mutants in the presence of sorbate and acetate

The growth of the same five tryptophan biosynthetic pathway mutants were compared to the BY parent strain in the presence of increasing concentrations of sorbic acid (0, 0.45, 0.9 and 1.8 mM) and acetic acid (0, 40 and 80 mM). The weak

acid preservatives were added to growing cultures in YPD medium at pH 4.5 with and without the presence of tryptophan supplementation and a comparison of growth inhibition for each of the strains was conducted, changes in growth were measured using the Bioscreen turbidometer.

Figures 5.6 a) and b) show the results for bioscreen analysis of the cultures BY4741 (wild type) and the *trp5*Δ mutant cells only at pH 4.5 in the presence of either a) 0.9 mM or b) 1.8 mM sorbic acid, with and without tryptophan supplementation. The graphs represent the analysis of three data sets, with the plots also showing calculated standard deviations for the data.

The results showed that the *trp5*Δ mutant strain was sensitive to the sorbate stress compared to the growth of the wild type. However, sorbate hypersensitivity was suppressed with the addition of a high level of tryptophan to the growth medium. The supplementation almost restored the growth of the *trp5*Δ mutant to the level of the sorbate stressed wild type cells (*TRP*$^+$ BY4741) lacking such tryptophan addition.

Figure 5.7 shows the results for the bioscreen analysis of the cultures BY4741 wild type a) and c) and its *trp5*Δ mutant derivative b) and d), in the presence of 0, 40 and 80 mM acetate. The cultures in a) and b) were grown in the absence of any medium supplement, whereas the cultures in c) and d) were grown with the addition of 50 mM tryptophan. The resultant growth plots show that the *trp5*Δ mutant is clearly much more sensitive to acetate than the wild type under these conditions (comparing Figure 5.7 a) and b)). The *trp1*Δ, *trp2*Δ, *trp3*Δ and *trp4*Δ mutants were also compromised under these conditions (results not shown), their growth rates being essentially similar to that of the *trp5*Δ mutant cells shown in Figure 5.7 b).

The *trp5*Δ mutant is extremely acetate-sensitive, but this sensitivity is almost totally suppressed by a high level of tryptophan addition (comparing Fig. 5.7 b) and d)). Such tryptophan supplementation restored the growth of the acetate-stressed *trp5*Δ mutant cells to that of control, acetate-stressed wild type (BY) cells lacking such supplementation (comparing Fig. 5.7 a) and d)).

5.2.6. Investigations into the effect of over-expression of Tat2p on sorbate resistance in yeast *trp-* mutants

To investigate further weak acid resistance in *trp-* mutants, Bauer *et al.* (2003) studied the effects of over-expression of the high affinity tryptophan permease, Tat2p. The *TAT2* gene was placed under the control of the strong, constitutive *ADH1* promoter in the multicopy vector pAD4M (Ballester *et al.*, 1989), yielding the plasmid pTAT2. This construct and the empty vector pAD4M were then introduced into both *trp1-Δ63* and *TRP1* (TRP^+) versions of strain YPH499. Undiluted overnight cultures of the indicated transformants were serially diluted, pinned onto YPD agar at pH 4.5 containing increasing concentrations of sorbic acid (0, 1, 2, 2.25 and 2.5 mM).

Figure 5.8 a) shows that the increased sorbate sensitivity due to *trp1-Δ63* was substantially suppressed by the p*TAT2* over-expression vector, a plasmid which can almost restore the growth of the sorbate-stressed *trp1-Δ63* cells to the level of growth displayed by an isogenic TRP^+ prototroph.

Bauer, B. *et al.* (2003) also transformed a mutant defective in the War1p transcription factor (*war1-42*) and its YPH499 parent (both *trp1-Δ63* strains) with pTAT2 and the empty pAD4M vector. Transformants were then tested for their ability to grow in the presence of sorbate. Again undiluted overnight cultures of the indicated transformants were serially diluted, pinned onto YPD agar at pH 4.5 containing increasing concentrations of sorbic acid (0, 0.5, 0.75 and 1 mM). Figure 5.8 b) shows that the pAD4M strain could not sustain the growth of the *war1-42* mutant on 0.75 mM sorbate, but that p*TAT2* could enable these cells to tolerate sorbate at up to 1 mM.

5.2.7. Investigation into the loss of Pdr12p, Azr1p and the effect of a double deletion mutant *Δpdr12 Δazr1* to determine the effect on acetate resistance

Work by a collaborative group (Piper *et al.*, 1998) found contradictory results when studying acetate resistance of two mutant strains *Δpdr12* and *Δazr1*. The group constructed a double *Δpdr12 Δazr1* mutant to determine which of the proteins,

Pdr12p or Azr1p, were more important for acetate resistance (Bauer *et al.*, 2003). The resistances of the double deletion mutant along with the corresponding Δ*pdr12* and Δ*azr1* single gene deletes were investigated in the presence of acetate and sorbate.

Figure 5.9 shows a photograph of the resultant growth after 3 days on YPD agar plates at pH 4.5 containing increasing levels of sorbic and acetic acid. The strains tested were, wild type (FY1679-11c - W+), Δ*pdr12* (FY809), Δ*azr1* (*11c-azr1*Δ), both single mutants and also a Δ*pdr12* Δ*azr1* double mutant (all isogenic to FY1679-11c). Results are courtesy of Bauer *et al.* (2003).

The loss of Azr1p did not result in any increased sensitivity to either of the acids tested (all of the mutant strains contained no auxotrophic requirements for aromatic amino acids). In addition, the loss of Pdr12p alone in this background resulted in slightly increased resistance to acetate. However, the results appeared to indicate that acetate and sorbate sensitivities were being enhanced with the loss of both Azr1p and Pdr12p (i.e. the Δ*pdr12* Δ*azr1* double mutant).

5.2.8. Investigation of the loss of *PDR12* in different parental backgrounds to determine the effect on acetate resistance

Further studies by Bauer *et al.* (2003) investigated the effects of high levels of Pdr12p induction on acetate resistance, using the strain *GAL1-PDR12*. This *PDR12* gene was not under the control of its normal promoter, but instead controlled by the *GAL1* promoter. The wild type (W+, FY1679-28c) and the *GAL1-PDR12* were plated onto YPD (glucose) and YPGalactose agar at pH 4.5 containing increasing concentrations of acetate (70 to 90 mM). Both strains were added to the plates using overnight cultures diluted to neat, 1 in 10 and 1 in 100 serial dilutions.

Figure 5.10 shows a photograph of the resultant growth after 3 days incubation at 30°C. Growth of the wild type yeast (W+) on galactose compared to glucose was reduced over the acetate range and thus this strain more sensitive to the acetate on galactose media. The strong Pdr12p induction with the growth of the *GAL1-PDR12*

strain on galactose increases this acetate sensitivity still further (thus decreasing acetate resistance). Results courtesy of Bauer *et al.* (2003).

5.2.9. Investigations into the effects of losing Pdr12p and Azr1p on acetate resistance

A *TRP*$^+$ wild type, Δ*pdr12* and Δ*azr1* single mutants and a Δ*pdr12* Δ*azr1* double mutant were grown in the presence of 0 mM, 0.9 mM sorbic acid and 80 and 120 mM acetic acid. The weak acid preservatives were added to growing cultures in YPD medium at pH 4.5. A comparison of growth was conducted using the Bioscreen turbidometer.

The results in Figure 5.11 confirm that the loss of Azr1p alone does not increase the sorbate sensitivity of *TRP*$^+$ cells, though this loss slightly increased the sorbate sensitivity of the Δ*pdr12* mutant (Fig. 5.11 b)). The Δ*pdr12* and Δ*azr1* single gene deletes grew considerably better than the wild type at pH 4.5 in the presence of 120 mM acetate (Fig. 5.11 d)). After an extended lag phase, the Δ*pdr12* Δ*azr1* double mutant also grew considerably better than the wild type.

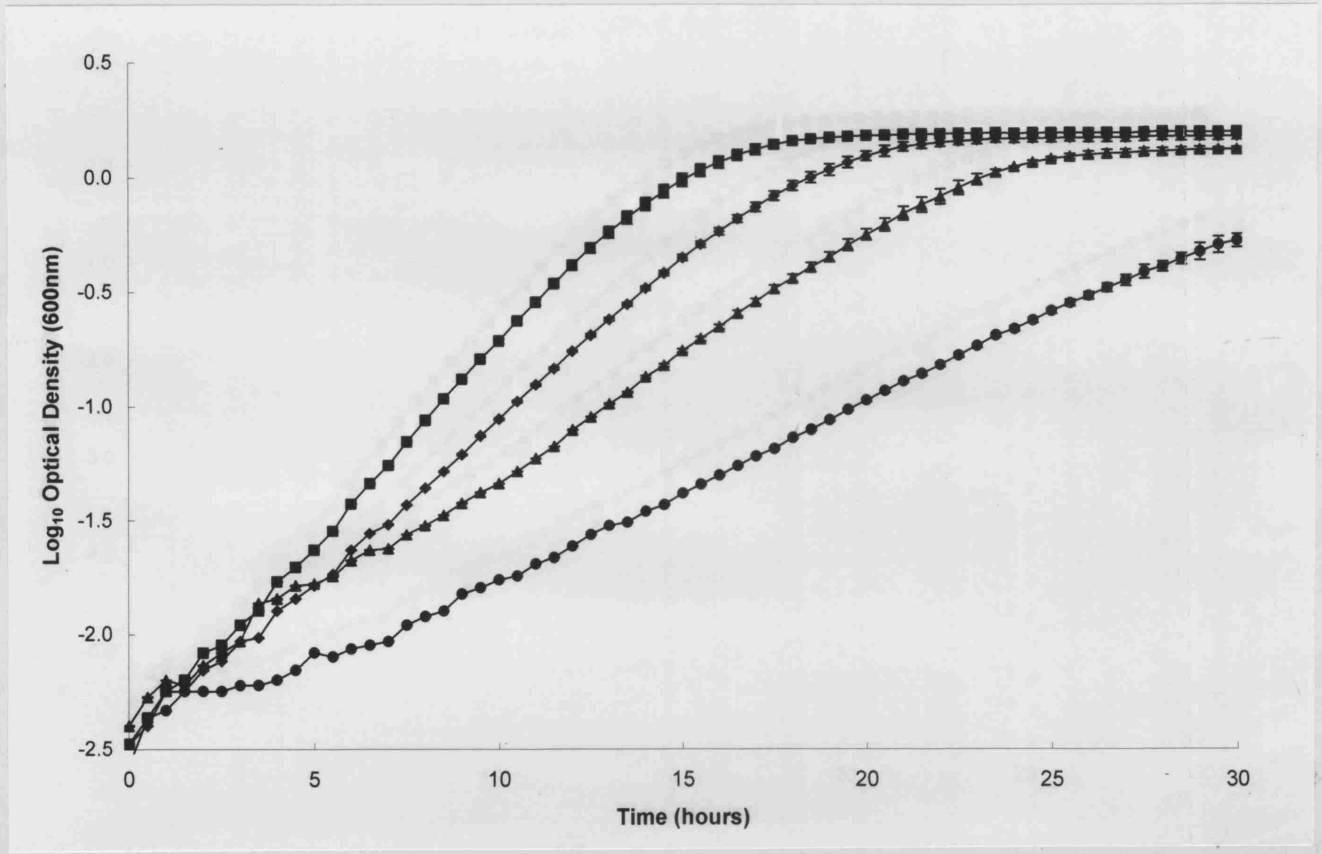


Figure 5.1

The effect of increasing sorbate concentrations 0 mM (■), 0.45 mM (▲), 0.9 mM (◆) and 1.8 mM (●) on the growth of *S. cerevisiae* BY4741 in YPD media at pH 4.5 measuring changes in logarithmic optical density (600nm) over time (hours), with calculated standard deviations.

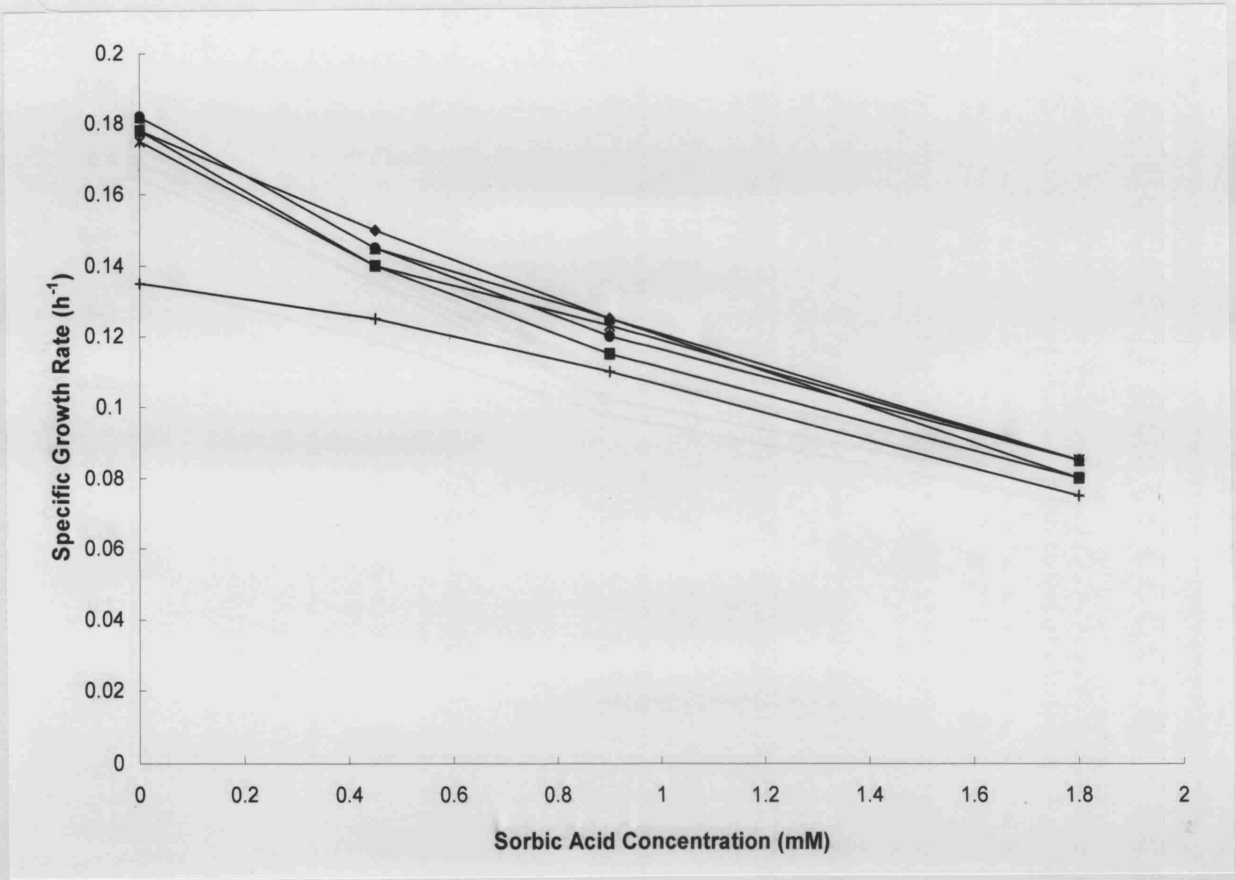
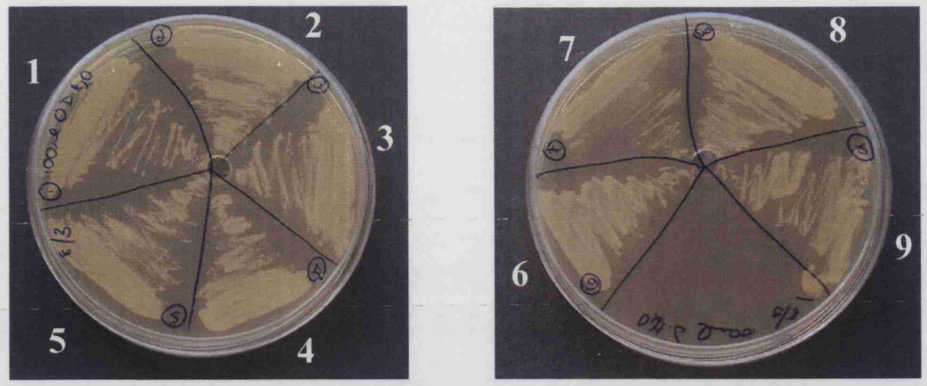


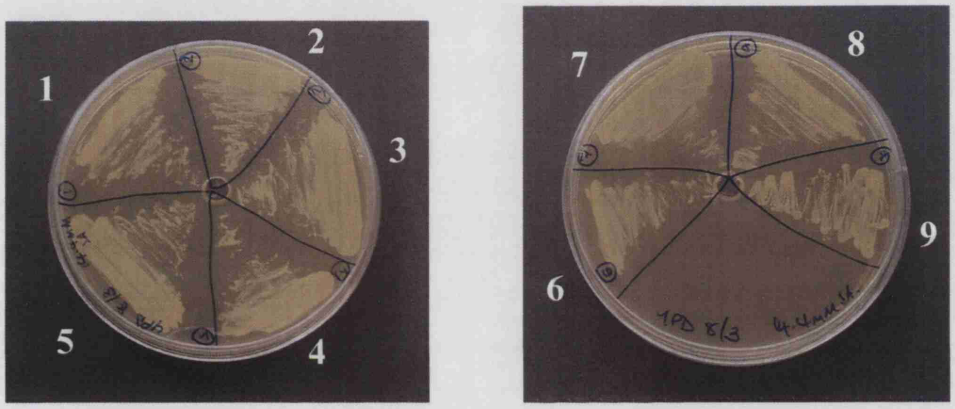
Figure 5.2

The effect of increasing concentrations of sorbic acid (mM) on growth rate (h^{-1}) for the *S. cerevisiae* parent strain BY4741 (■) and deletion mutant strains *ach1* (◆), *hsp26* (▲), *cph1* (●), *cys4* (✶), and *hcr1* (+) in YPD media at pH 4.5.

a)



b)



c)

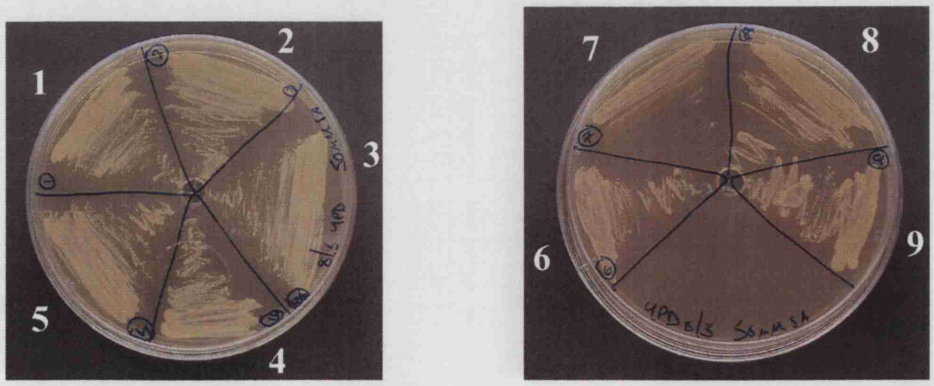


Figure 5.3 a), b) and c)

Growth of *S. cerevisiae* strains on YPD zonal inhibition agar plates depicting a) 0 mM, b) 14.4 mM and c) 50 mM sorbic acid. The *S. cerevisiae* strains are numbered 1. BY4741 (a parent), 2. BY4743 (diploid a /α parent), 3. *ach1*, 4. *hsp26*, 5. *cph1*, 6. unrelated test strain 7. *cys4*, 8. *ylr192c* and 9. unrelated test strain.

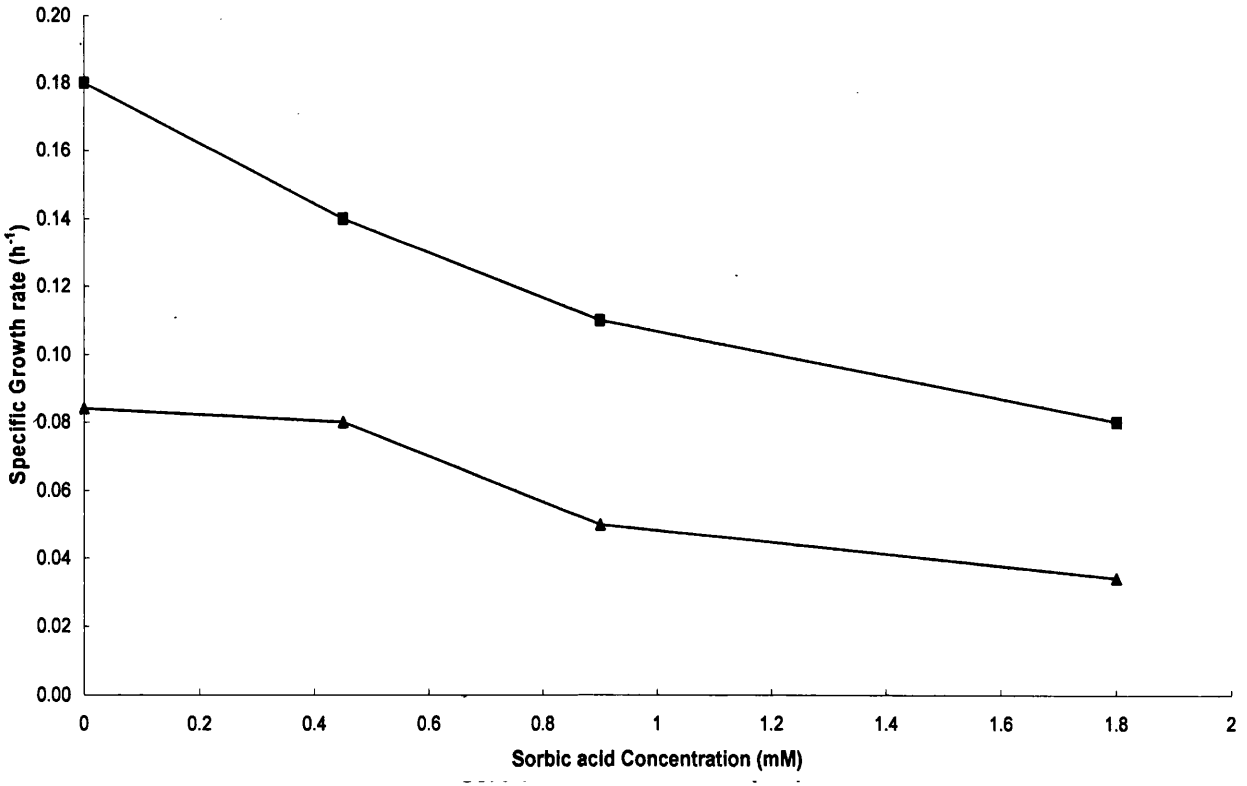


Figure 5.4

The effect of increasing concentrations of sorbic acid (mM) on the growth rate (h^{-1}) of the *S. cerevisiae* parental strains BY4741 (*MAT*a) (■) and FY1679-28c (*MAT*a) (▲) in YPD at pH 4.5.

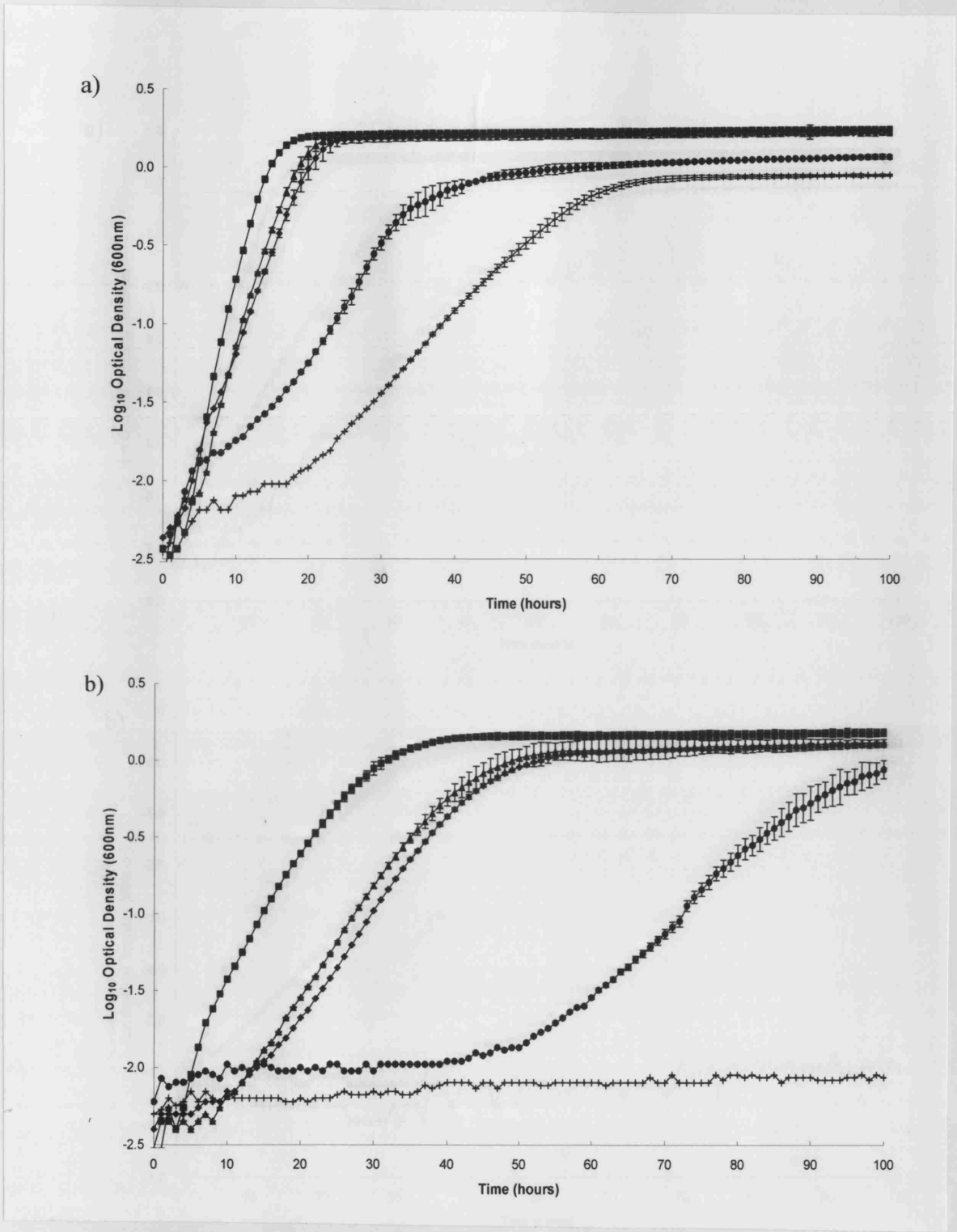


Figure 5.5 a) and b)

Growth of *S. cerevisiae* strains a) BY4741 and b) *trp5*Δ mutant in the absence (■), or the presence of increasing concentrations of sorbic acid (0.45 mM (▲), 0.9 mM (♦), 1.8 mM (●) and 3.6 mM (+)) in YPD pH 4.5. Measuring changes in logarithmic optical density (600nm) over time (hours), with calculated standard deviations.

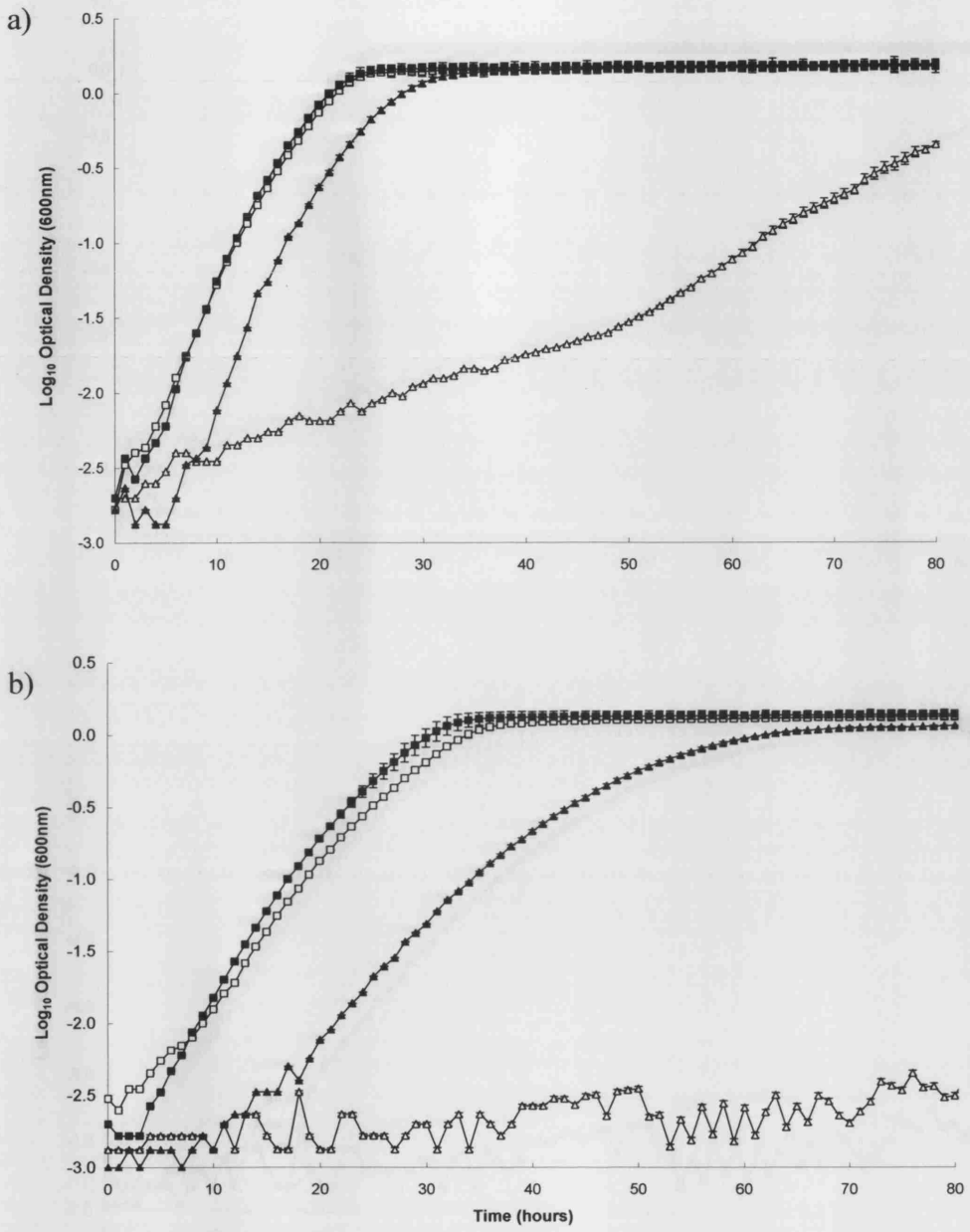


Figure 5.6 a) and b)

Bioscreen culture of BY4741 wild type (□, ■) and *trp5*Δ mutant cells (▲, Δ) at pH 4.5 in the presence of either a) 0.9 mM or b) 1.8 mM sorbic acid, either in the absence (□, Δ) or the presence (■, ▲) of 50 mM tryptophan supplementation. Measuring changes in logarithmic optical density (600nm) over time (hours), with calculated standard deviations.

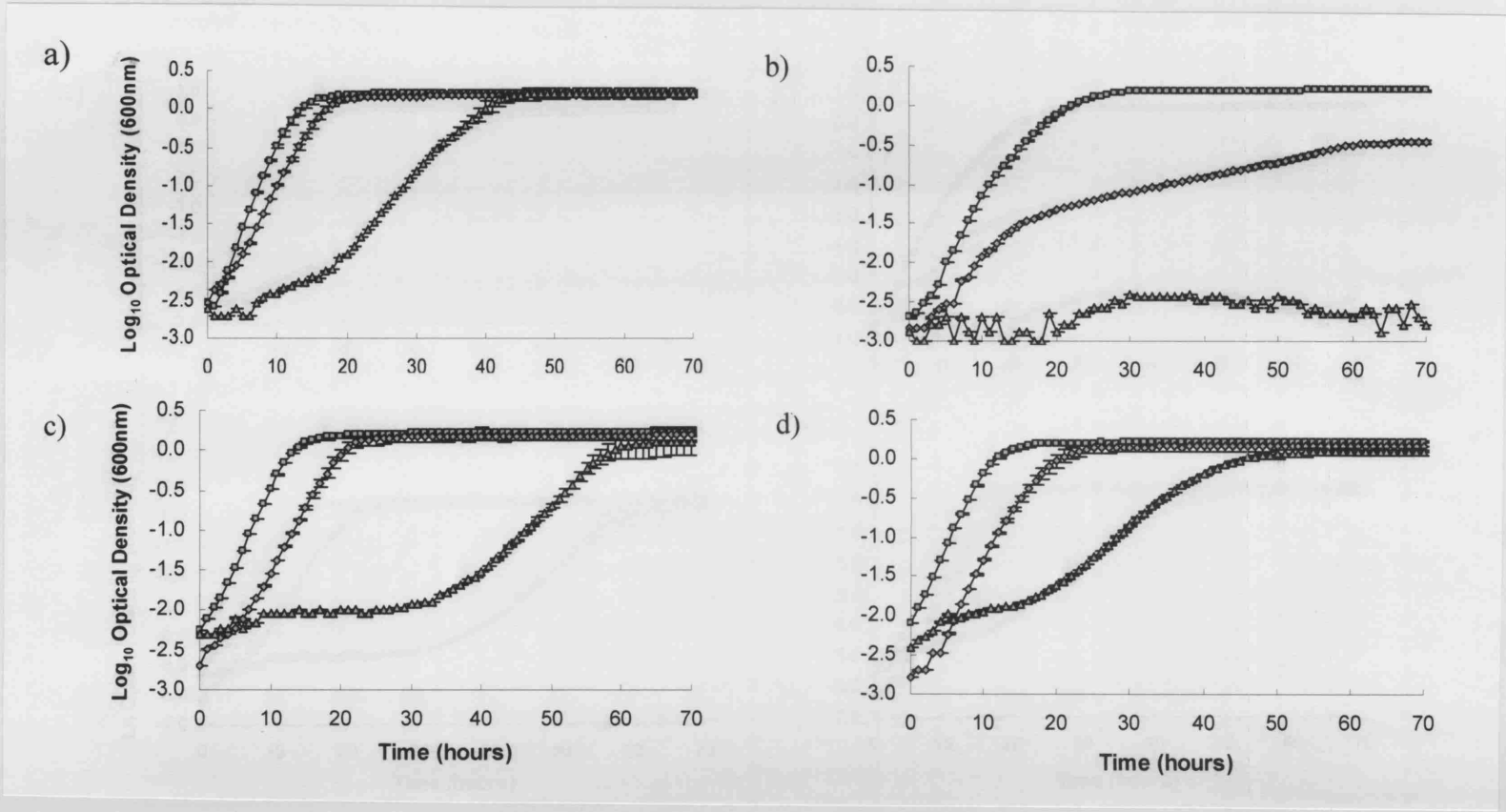
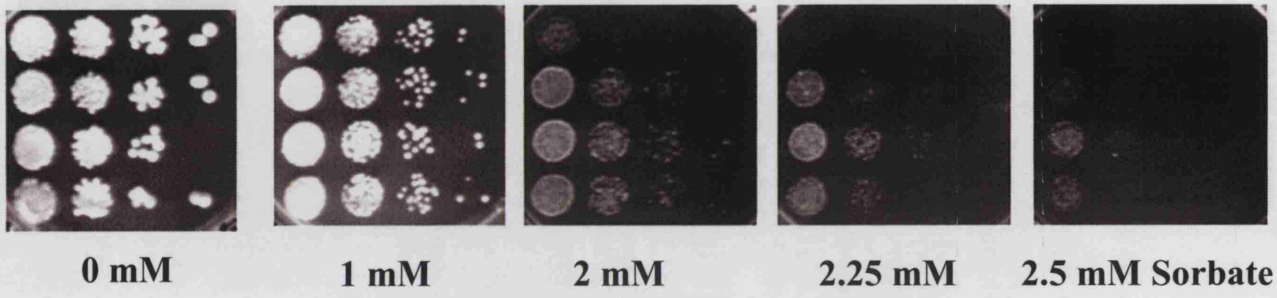


Figure 5.7 a), b), c) and d)

The effect of growth of *S. cerevisiae* strains *TRP*$^{+}$ BY4741 wild type a) and c) and *trp5*Δ mutant derivative b) and d) in the presence of no weak acid (■), 40 mM acetate (◆) and 80 mM acetate (▲). The cultures a) and b) were grown in the absence of any medium supplement, cultures c) and d) were grown with 50 mM tryptophan supplementation. Changes in growth were measured using the Bioscreen (logarithmic O.D. $_{600}$) over time (hours), with calculated standard deviations.

a) **YPH499 pAD4M**
YPH499 p*TAT2*
YPH499 TRP pAD4M
YPH499 TRP p*TAT2*



b)

OD 0.2 YPH499	OD 0.02 YPH499	OD 0.2 42-1	OD 0.02 42-1
42-1 pAD4M	42-1 pAD4M	42-1 p*TAT2*	42-1 p*TAT2*
42-1 pAD4M	42-1 pAD4M	42-1 p*TAT2*	42-1 p*TAT2*
42-1 pAD4M	42-1 pAD4M	42-1 p*TAT2*	42-1 p*TAT2*

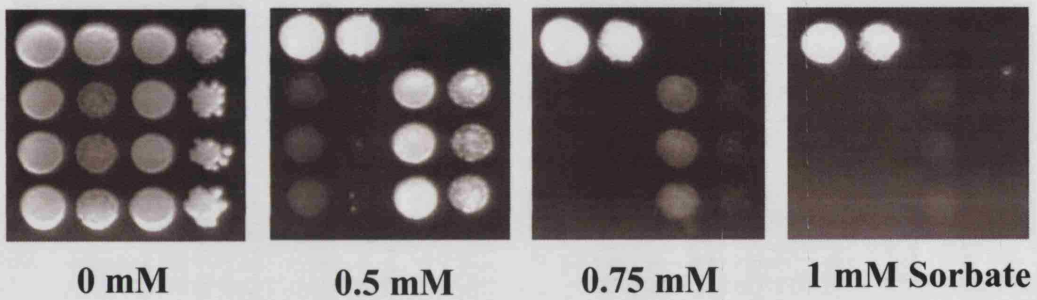


Figure 5.8 a) and b)

a) The effects of Tat2p over-expression on sorbate resistance

b) Investigating sorbate sensitivity in a mutant (*war1-42*) defective in Pdr12p induction.

In both cases undiluted overnight cultures of the indicated transformants were serially diluted, pinned onto pH 4.5 YPD agar and the plates incubated 3 days at 30^0C. Results courtesy of Bauer *et al.* (2003).

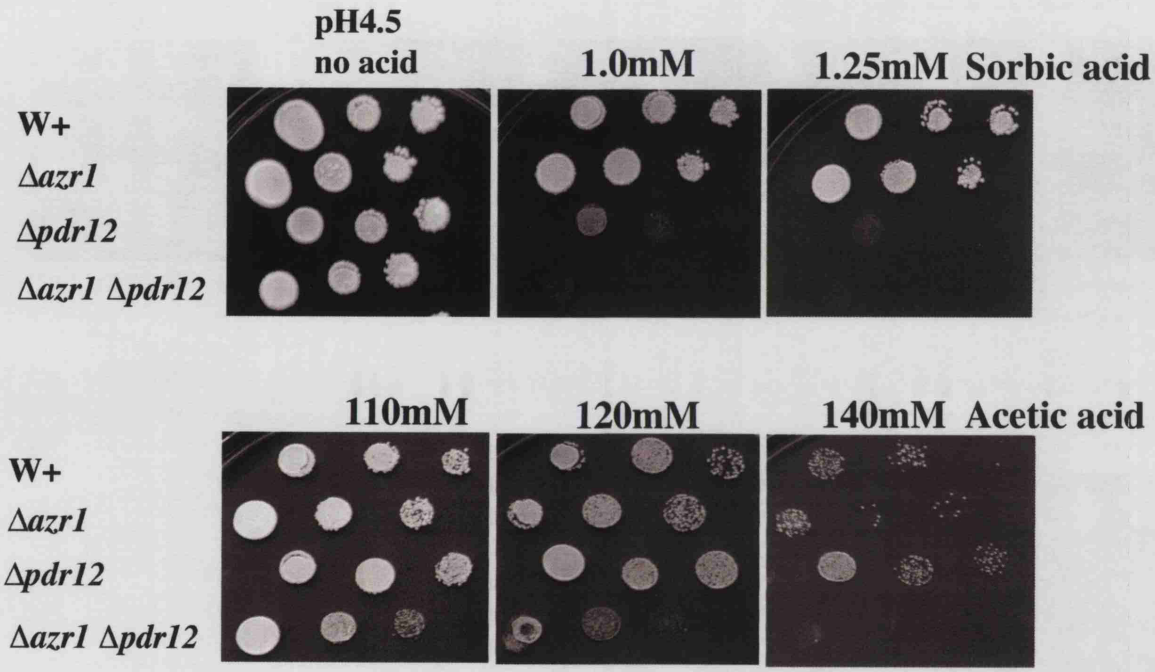


Figure 5.9

Growth on YPD agar pH 4.5 plates, photographed after 3 days at 30^0C, of W+ wild type (FY1679-11c), Δ*pdr12* (FY809), Δ*azr1* (11c-azr1Δ), both single mutants and Δ*pdr12* Δ*azr1* double mutant (all isogenic to FY1679-11c), in the presence of the indicated levels of sorbic acid or acetic acid. Each set of three spots comprised of an undiluted overnight culture, 1 in 10 and 1 in 100 serial dilutions.

Results courtesy of Bauer *et al.* (2003).

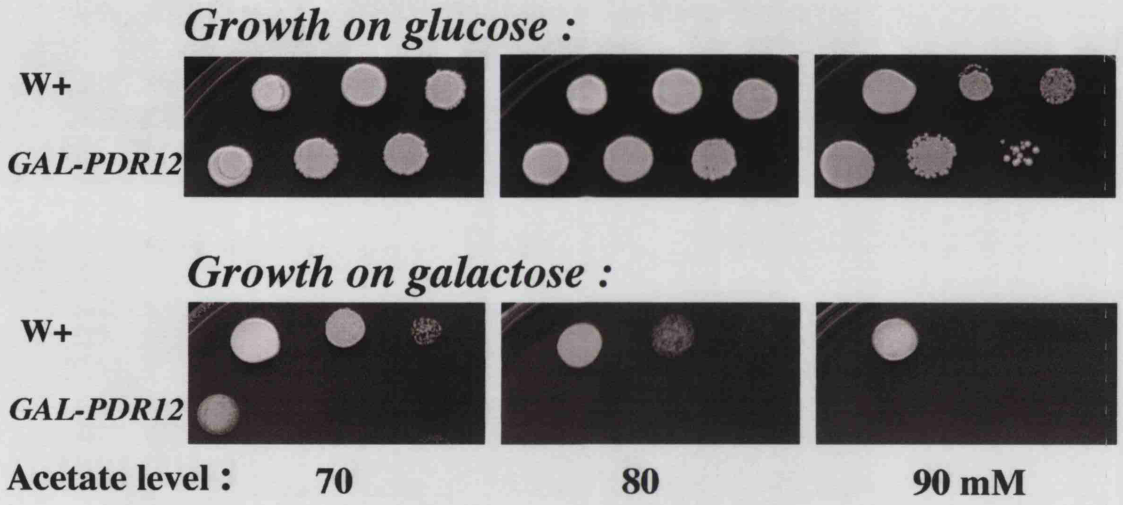


Figure 5.10

Growth of wild type (W+; FY1679-28c) and *GAL1-PDR12* cells on YPD (glucose) and YPGalactose in the presence of acetic acid. Undiluted, 1 in 10 and 1 in 100 serial dilutions of overnight cultures of both strains were added to pH 4.5 agar containing the indicated level of acetic acid. The photograph was taken 3 days after incubation at 30°C. Photograph courtesy of Bauer *et al.* (2003).

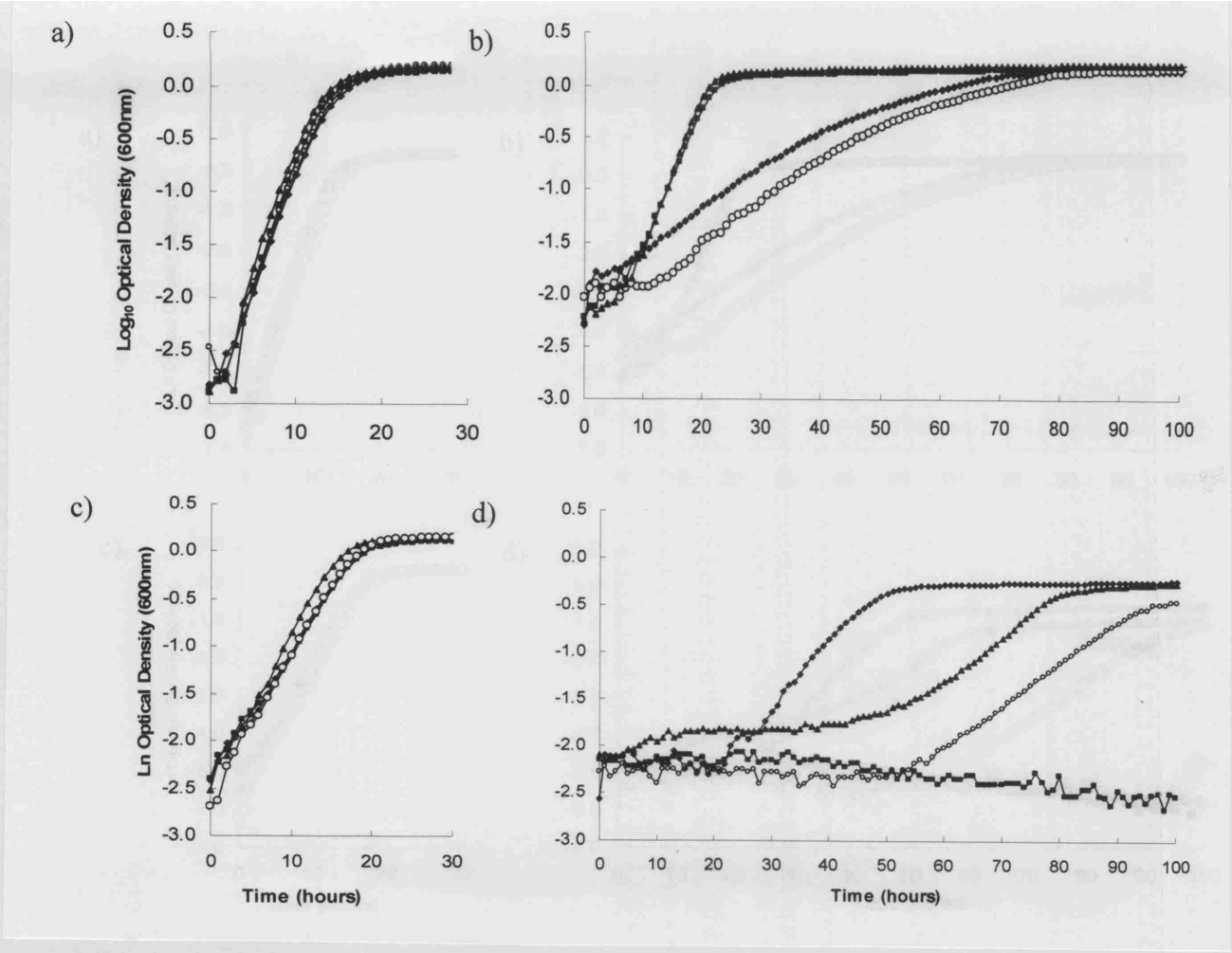


Figure 5.11 a), b), c) and d)

The effect of growth of *S. cerevisiae* strains *TRP*$^{+}$ wild type (■), Δ*pdr12* (◆) and Δ*azr1* (▲) single mutants and a Δ*pdr12* Δ*azr1* double mutant (○) in YPD at pH 4.5 in the presence of a) no stress agent, b) 0.9 mM sorbate, c) 80 mM acetate and d) 120 mM acetate. Changes in growth were measured using the Bioscreen (logarithmic O.D. $_{600}$) over time (hours).

5.3. Discussion

In the previous Chapter (Chapter 4) proteomic studies were conducted to identify key protein that were either induced or repressed as a result of weak acid stress on the yeast cells S*accharomyces cerevisiae*. These proteomic studies a possible eleven cytosolic proteins that were up-regulated in response to sorbate stress. Of this group, only five were chosen for further phenotypic analysis as having either relevance in the literature or as a result of other researcher's findings to be relevant to weak acid resistance.

5.3.1. Phenotypic analysis of deletion mutants

This Chapter continued the investigation of the five chosen proteins by phenotypic evaluation of gene deletion mutants using strains from the *Saccharomyces* Genome Deletion collection. The required deletion mutant strains were only available from this collection in a BY genetic background (haploid parental strain BY4741) which is almost genetically identical to that of the FY parental strain used in the proteomic (Chapter 4) and pH_i studies (Chapter 3), although the only difference was that the BY strain was TRP^+ and the FY strain *trp-*.

Contrary to expectation, the phenotypic evaluation of these gene deletion mutants revealed that none were sensitive to sorbic acid. Two of the deletion strains, *hcr1* (a gene without known function) and *cys4* (a mutant defective in biosynthesis of cysteine) showed very slight sensitivities to the weak acid, however, these results were not consistent across the tests performed. The mutant strain *hcr1* showed an increased level of sensitivity compared to the other strains when grown in the presence of the weak acid in liquid media, however, the amount of inhibition was similar to the other strains as the concentration of sorbate increased (Figure 5.2). However, on the agar inhibition plates with the highest concentration of weak acid the *cys4* mutant was slightly more acid sensitive, but the *hcr1* mutant was not (Figure 5.3). It can be therefore concluded that these findings are inconclusive and have some inconsistencies between the results for weak acid sensitivity.

In Chapter 4, when comparing the results from this proteomic study with those of other researchers, some the identified proteins from this study correlated well with these other findings, thus providing the basis and confidence in the choices. However, from this list *hcr1* had not been identified by the other studies, thus perhaps it was not surprising that this mutant was not sorbate sensitive. However, it was surprising that the other four chosen deletion mutants (*ach1*, *cys4*, *hsp26* and *cph1*) did not show any weak acid sensitivity when compared to the parent. Furthermore, these results did not agree with other findings (Mollapour *et al.*, 2004, de Nobel *et al.*, 2001 and Schuller *et al.*, 2004) that did show sorbate sensitivity for these deletion mutants.

Thus it can be concluded the results from this study, the mutants tested did not show any sorbate sensitivity, this conflicting with results from other studies. Therefore, further investigations would be required to determine the reasons for these inconsistences with the deletion mutant strains. However, these investigations would not be performed within this thesis study due to the direction of study taking a different route.

5.3.2. Differences in sorbate sensitivity between the FY and BY parents

Results from experiments conducted within this study also discovered that two genetically almost identical strains FY1629-28c and BY4741 when compared had two very different sorbate sensitivities. When screening the two parents in liquid culture containing sorbic acid, the FY strain was considerably more sorbate sensitive than the BY strain, the addition of sorbate significantly inhibiting the growth of the FY strain compared to that of BY (Figure 5.4). The only major difference between these strains is their genetic backgrounds, FY1629-28c is a tryptophan auxotroph (*trp-*) and BY4741 a tryptophan phototroph (TRP^+). Thus this difference was investigated further to whether auxotrophy for tryptophan could be enhancing this observed weak acid sensitivity.

This result also raised questions with regard to the use of an already sorbate sensitive FY parent in the proteomics study in Chapter 4, could have possibly given different and heightened expression responses that may not have been seen in the BY parent.

5.3.3. Investigations to determine the sorbate sensitivity of a series of tryptophan auxotrophic mutants

To investigate the effects of defects in tryptophan biosynthesis on weak acid resistance, five deletion mutants for enzymes of the tryptophan biosynthesis pathway were obtained from the EUROSCRAF collection in the BY4741 background. These mutants were studied in the presence of both sorbate and acetate weak acid stress. Sample results for the *trp5*Δ mutant compared to the BY parent are depicted in Figure 5.5 and similar results were obtained *trp1*Δ, *trp2*Δ, *trp3*Δ and *trp4*Δ, showing that all were sensitive to sorbate, when compared to the BY parent. All of these mutants, unlike the BY parent, need to catalyse the uptake of tryptophan from the medium in order to grow, as one steps in their tryptophan biosynthesis pathway is blocked.

Suspecting that the weak acids were strongly inhibiting the uptake of tryptophan, the effects of supplementing the medium with high levels of tryptophan was investigated. Whilst the *trp5*Δ mutant was extremely sorbate sensitive, this hypersensitivity was almost totally suppressed by the high levels of tryptophan (Figure 5.6). Such supplementation restored the growth of the weak acid stressed *trp5*Δ mutant cells to that of the control, stressed wild type BY (*TRP*$^+$) cells lacking such supplementation.

Similar experiments were repeated using the same tryptophan mutant strain with and without the addition of acetate and again, this hypersensitivity was substantially suppressed with the addition of high levels of tryptophan supplementation to the medium. As in the previous result this supplementation almost restored the growth of the *trp5*Δ mutant to that of acetate-stressed wild type cells lacking such amino acids (Figure 5.7). Thus concluding that indeed the tryptophan biosynthetic mutants are weak acid sensitive, but this sensitivity can be restored by adding extra tryptophan.

These findings that the *trp1* to *5*Δ mutants were weak acid sensitive was also in agreement with the later findings of Mollapour *et al.* (2004) and Schuller *et al.* (2004) who both identified the tryptophan biosynthesis deletion mutants as being sorbate sensitive (both conducting their studies in the BY (*TRP*$^+$) background).

However, the studies of de Nobel *et al.* (2001) did not identify any of these *trp-* mutants, the fact that they conducted the screen in the more sorbic acid sensitive FY *trp-* parent genetic background could provide an explanation in the differing results.

5.3.4. Over-expression of tryptophan permease can suppress weak acid sensitivity in tryptophan auxotrophs

The above results revealed that a requirement for uptake of aromatic amino acids from the culture medium lead to unusually high sensitivity to weak organic acid stress. Thus it was hypothesised that an increased capacity for the *trp-* cells to catalyse uptake of aromatic amino acids might therefore suppress this sensitivity. To obtain further evidence for this hypothesis, Bauer *et al.* (2003) studied the effects of over-expressing the high affinity tryptophan permease, Tat2p (Schmidt *et al.*, 1994). The increased sorbate sensitivity due to *trp1-Δ63* was substantially suppressed by the p*TAT2* over-expression vector, a plasmid which could almost restore the growth of the sorbate-stressed *trp1-Δ63* cells to the level of growth displayed by an isogenic TRP^+ prototroph (Fig. 5.8 a)).

5.3.5. Determination of differing cellular responses to weak acids of the same deletion mutants be due to tryptophan auxotrophy

Research by the group had given contradictory results when studying acetate sensitivity in *pdr12* and *azr1* mutants. Earlier work had shown that the mutant strain *pdr12Δ* was more acetate sensitive than the wild type (Piper *et al.*, 1998) and a separate study had reported that Azr1p, a plasma membrane transporter of the major facilitator superfamily, also conferred acetate resistance (Tenreiro *et al.*, 2000).

Later studies using a different strain background which contained no auxotrophic requirements for aromatic amino acids (i.e. were TRP^+) showed this time the loss of Pdr12p or Azr1p resulted in no increased sensitivity to acetate. Instead, the loss of Pdr12p alone in this background resulted in slightly increased resistance to acetate (Bauer *et al.*, 2003).

This Chapter describes part of a study initiated to investigate these findings further, and to determine which of these proteins, Pdr12p or Azr1p, were more important for acetate resistance (Bauer *et al.*, 2003) due to the observation that losses of Pdr12p or of Azr1p appeared to be exerting opposite effects on acetate resistance in *trp-* and TRP^+ genetic backgrounds.

Studies were conducted to investigate the effects of the loss of Pdr12p and Azr1p on acetate resistance in TRP^+ cells. The results in Figure 5.9 show that the losses of these genes result in either neutral effects or a decreased sensitivity to acetate in TRP^+ cells, whereas in *trp-* mutants the losses of these transporters increased sensitivity to acetate.

To study these results further Bauer *et al.* (2003) investigated the effects of high levels of Pdr12p induction on acetate resistance by using the strain *GAL1-PDR12*, where the *PDR12* gene was placed under the control of the *GAL1* promoter because the *PDR12* gene is not normally induced by acetate (Hatzixanthis *et al.*, 2003). However, during growth of this *GAL1-PDR12* strain on galactose media, *PDR12* can be induced to levels similar as to those seen in wild type cells exposed to sorbic acid stress (Hatzixanthis *et al.*, 2003). Results in Figure 5.10 showed that growth of normal wild type yeast on galactose relative to glucose slightly decreased acetate resistance, yet the strong Pdr12p induction with the growth of the *GAL1-PDR12* strain on galactose decreases acetate resistance still further. This result contrasts greatly with that for sorbate, where an increased resistance results from the same *GAL1* promoter-directed induction of Pdr12p (Hatzixanthis *et al.*, 2003). This provided further evidence that the induction of Pdr12p, though beneficial for resistance to C_{3-8} aliphatic carboxylic acids and to sorbate (Piper *et al.*, 1998 and Hatzixanthis *et al.*, 2003), is actually somewhat detrimental for resistance to acetate. Unpublished work by M. Mollapour has shown that neither Pdr12p nor Azr1p is actually induced by acetate stress although a *GAL1* promoter-directed induction of Pdr12p clearly results in a reduced resistance to acetate (Figure 5.10).

These results appeared to contradict the earlier findings by the group that loss of Pdr12p resulted in a decrease in acetate resistance (Piper *et al.*, 1998). However, the strains used in these earlier studies were identical to those used to obtain the data in

Figure 5.9 and 5.10, except that they possessed the additional *trp1-Δ63* mutation. When the *trp*1-*Δ63* strains used in earlier work were re-tested (Piper *et al.*, 1998), they were found to be generally less weak acid resistant than the corresponding TRP^+ strains and thus explaining these observed differences.

Further control experiments (Bauer *et al.*, 2003) showed that the high tryptophan levels had no effect on Pdr12p induction by sorbate (results not shown). Furthermore, because exogenous tryptophan suppresses both the sorbate (Figure 5.6) and acetate (Figure 5.7) and sorbate (Figure 5.8) sensitivities of *trp-* biosynthetic mutant cells (only the sorbate is a Pdr12p inducer) it is clear that this sensitivity suppression by exogenous tryptophan bears no relationship to the weak acid inducibility of Pdr12p. Furthermore, Pdr12p levels were not affected by *TAT2* over-expression, indicating that this rescue of acid-stressed cells by pTAT2 acts independently of the Pdr12p transporter. Tat2p over-expression could partially rescue the sorbate sensitivity of the *war1-42* mutant, a mutant lacking Pdr12p induction, in a *trp1* genetic background (Figure 5.8 b)).

Bioscreen analysis of *Δpdr12* and *Δazr1* single gene deletes in the TRP^+ backgrounds (Figure 5.11) confirmed that loss of Azr1p alone did not increase the sorbate sensitivity of TRP^+ cells, though this loss increased the sorbate sensitivity of the *Δpdr12* mutant (Figure 5.11 b)). Unexpectedly, the *Δpdr12* and *Δazr1* single gene deletes grew considerably better than the wild type at pH 4.5 in the presence of 120 mM acetate (Figure 5.11 d)). After an extended lag phase, the *Δpdr12 Δazr1* double mutant also grew considerably better than the wild type. Loss of either of Pdr12p or Azr1p appears therefore to be beneficial for growth in the presence of high concentrations of acetate. The reasons for this improved growth are not clear, but one possibility is that these mutants are not displaying the apoptotic events normally seen in yeast cells treated with high levels of acetate (Ludovico *et al.*, 2001). It is therefore unlikely that Pdr12p or Azr1p confer acetate resistance as suggested by earlier work (Piper *et al.*, 1998) indeed it appears that their loss may actually elevate this resistance (Figure 5.11).

In summary, it has been shown that aromatic amino acid auxotrophic strains are hypersensitive to sorbic acid, but this sensitivity can be repressed by either supplementing extra tryptophan to the medium or the over-expression of a tryptophan permease in such *trp-* cells. This hypersensitivity has clearly caused conflicting data when comparing deletion mutants in different genetic backgrounds, the *trp-* background giving results indicating possible weak acid sensitivity where there is not.

The proteins identified in Chapter 4 proteomic study were done so using an FY parent strain that was *trp-* and the induced proteins identified as a result of this stress, could possibly not be sensitive to sorbate in a TRP^+ background. However, the results from Chapter 4 were compared to studies (Mollapour *et al.*, 2004 and Schuller *et al.*, 2004) that conducted similar sorbic acid stress analysis in BY TRP^+ background, thus giving confidence in the findings. However, for the work of de Nobel *et al.* (2001) this was not the case and had used the same FY *trp-* strain as in Chapter 4. In particular there was only one protein that was only identified by de Nobel *et al.* (2001) and this was *cph1* (cycophilin peptidyl propyl cis-trans isomerase) and it is possible that for this deletion mutant in particular the *trp-* background gave it a hypersensitive phenotype that was not seen in the TRP^+ background.

The differences in the patterns of gene expression seen Mollapour *et al.* (2004) compared to the results of de Nobel *et al.* (2001) were commented upon. It was hypothesized that the differences could be due to the work of de Nobel was investigating adaptation over a longer period of time rather than that of resistance over a short period of time. However, Mollapour *et al.* (2004) alluded to a possible issue with conducting such studies in a *trp-* background could give arise to different expression patterns. In deed this study confirms the hypothesis that such a *trp-* background would give rise to a set of genes sensitive to weak acid that would not normally be so under TRP^+ conditions. These results thus explaining the lack of correspondence between the changes incurred as a result or weak acid stress in the two different genetic backgrounds.

With this result in mind the findings of *pdr12* mutants were assessed to determine if the weak acid sensitivity seen for this mutant with sorbate and acetate was the case. This study confirmed that Pdr12p is still an integral element in the response to resistance to sorbic acid, however, it is not required for acetate resistance which was concluded earlier in *trp-* background strains.

It is therefore expressed, that care is required when conducting any kind of stress assessment with deletion mutants. The choice of parent strain with regard to the choice of the genetic background is imperative so that it does not impose additional bias to the results.

Chapter 6 – Discussion and Conclusions

6.1. The successful development of a fluorescent in vivo intracellular pH method

To attempt to understand further the role of intracellular pH as a potential trigger within stress responses of yeast, an *in vivo* method for pH_i determination was first developed. Unlike previously discussed methods (section 3.1.2.), this fluorescent technique allows the rapid and non-invasive assay to determine the effectivity of antifungal compounds on the pH_i in growing cells. Chapter 3 describes the successful adaptation and development of a pH-dependent intracellular fluorescence technique using the novel fluorescent probe CF-SE loaded in the cytosol (first developed for bacteria by Breeuwer *et al.*, 1996) to determine pH_i in the spoilage yeast *S. cerevisiae*. This probe in particular has a major advantage over other carboxy fluorescein probes due an additional succinimidyl ester group (SE) which is believed to form conjugates with the aliphatic amines on proteins and other intracellular molecules in the cell (Haughland, 1996). This allows the use of such conjugated probes to study pH_i under conditions that might damage cell membranes and would otherwise lead to the complete leakage of the probe from the cells to the external environment (Breeuwer *et al.*, 1996).

Indeed experiments testing the newly developed methodology showed that the probe was successfully loaded (Figure 3.5) and was not immediately effluxed out of the cell. Any issues with probe leakage during experiments performed over extended time scales could be removed either by using low external pH media (form pH 4.3 downwards) or subtracting the background fluorescence values from any data, thus reducing any inaccurate determinations.
This methodology did however have a few negative drawbacks, these being the amount of samples that could be measured during an experiment and thus the number of replicates that could be performed. Due to this issue, many of the experiments depicted were performed over a number of days.

The effectiveness of the pH_i methodology was confirmed by measuring the pH_i of the *PMA1* wild type yeast cells in a buffer system (Figures 3.6 and 3.7) and was compared to other studies in the literature. The resultant values compared favourably

with a number of referenced studies using fluorescence microscopy (Cimprich, P. *et al.,* 1995 and Imai, T. and Ohno, T., 1995). Furthermore, the pH_i values reported in this study were approximately 1 pH unit lower than those measured by ^{31}P-NMR spectroscopy (Gillies *et al.*, 1981 and Coote *et al.*, 1994), confirming the findings of Cimprich *et al.* (1995). A possible explanation for these differences could be that the high cell densities employed in the NMR studies resulted in the cells being in a non-physiological state thus affecting their pH_i values. Thus giving confidence to the resultant pH_i values calculated both by the developed technique.

6.2. Comparison of a parent and reduced-expression membrane H^+-ATPase mutant in static and growing systems show marked differences in pH_i on sorbate addition

The weak-acid food preservative, sorbic acid is known to reduce the pH_i of yeast cells (Krebs *et al.*, 1983; Cole & Keenan, 1987 and Booth & Kroll, 1989). Upon the addition of sorbic acid to CF-SE loaded cells suspended in biological buffer, large instantaneous reductions in pH_i were detected, thus confirming the effectiveness of this pH_i methodology to be able to rapidly and non-invasively measure pH_i fluctuations in *S. cerevisiae* (Figures 3.6 and 3.7).

As a further validation of this methodology, the pH_i was measured in a reduced-expression membrane H^+-ATPase mutant (*pma1-205*) and compared to the values observed with the isogenic parent, *PMA1* (Figure 3.7). In yeast, the plasma-membrane H^+-ATPase plays a major role in the maintenance of pH_i homeostasis by coupling ATP hydrolysis to the expulsion of protons, generating a proton gradient (Serrano, 1984). Unsurprisingly, this enzyme has been shown to be critically involved in resistance to stress factors that disturb pH_i homeostasis, for example heat (Coote *et al.*, 1994) and sorbic acid (Holyoak *et al.*, 1996).

In further experiments, the addition of sorbic acid resulted in a large drop in pH_i in both strains, to similar pH_i values. The drop in pH_i was less in the reduced-expression H^+-ATPase mutant possibly because the initial pH_i value was reduced compared to the parent strain (Figure 3.7). The *pma1-205* mutant has been shown to have 66% less membrane H^+-ATPase activity compared to the parent (Vallejo &

Serrano, 1989) thus it can be concluded that the mutant is less able to maintain a higher pH_i in a more acidic environment, thus possibly explaining this observation. Furthermore, weak acid theory states that the same concentration of undissociated sorbic acid would enter both strains but would dissociate to a lesser extent in the mutant strain, due to the lower pH_i, thus resulting in a smaller overall drop in pH_i.

Following experiments in buffer where large pH_i changes were successfully measured after addition of sorbic acid to static yeast cells. The pH_i's of growing yeast cells of *PMA1* and *pma1-205* in growth media (Figure 3.8) were measured. As shown previously (Holyoak *et al.*, 1996), the reduced-expression of the membrane H^+-ATPase resulted in significant extension of the lag phase compared to the wild-type parent. Measurement of pH_i during the lag and growth phases of both strains revealed a lower pH_i in the membrane H^+-ATPase mutant, thus confirming the results observed in buffer. However, the reduced pH_i in the mutant does not appear to account for the extended lag phase since upon exit from lag, the growth rates of both strains were similar (shown previously by Holyoak *et al.*, 1996).

This would suggest that the mutant strain does not have to attain a certain value of pH_i similar to that in the parent before outgrowth from lag can occur. Therefore, it would seem that another physiological consequence of reduced-expression of the membrane H^+-ATPase other than reduced pH_i would account for the extended lag phase in this strain.

It is generally accepted that low cytoplasmic pH is detrimental to the microbial cell, causing disruption to many critical physiological functions, such as, the regulation of key metabolic enzymes and cell division. Indeed many studies have suggested that the principal inhibitory action of weak acids is reduction in pH_i (Booth & Kroll, 1989, Salmond *et al.*, 1984 and Cole & Keenan, 1987). However, it is clear from these studies that the inhibition of growth is not solely due to the disruption of pH_i by weak acids, and a number of other cellular targets must be involved.
Furthermore, exactly why reduction of pH_i inhibits growth is less clear and a number of hypotheses have been proposed. Krebs *et al.* (1983) concluded that reduction of pH_i in yeast induced by exposure to benzoic acid resulted in the direct inhibition of phosphofructokinase which reduced glycolytic flux, energy generation and,

ultimately, growth. Another proposed mechanism is that weak acid induced intracellular acidification results in the elimination of the proton gradient across the membrane and the inhibition of essential nutrient uptake, such as amino acid transport (Freese *et al.,* 1973). Despite these studies, the role of pH_i disruption in growth inhibition by weak acids remains unclear and further work in this thesis sought to clarify these ideas.

6.3. Comparison of the inhibitory effects of two different antifungal actives; sorbic acid and amphotericin B

Unlike the weak acids, the inhibitory action of amphotericin B is believed to be due to gross membrane disruption and channel formation, causing significant permeability of the yeast plasma membrane to protons (Palacios & Serrano, 1978). To determine the importance of membrane disruption and the effects upon pH_i homeostasis, the actives amphotericin and sorbic acid were compared.

The results in this study showed that the inhibitory activity of sorbic acid in growing yeast cells was dependent upon two factors. Firstly, the amount of growth inhibition increased as the concentration of membrane permeable undissociated weak acid increased (Figure 3.10 a)), thus confirming the observations of previous studies (Salmond *et al.,* 1984 and Holyoak, *et al.,* 1996). Secondly, the amount of growth inhibition was greater at lower culture pH values despite exposure to a constant concentration of undissociated sorbic acid (Figure 3.12 a)). Furthermore, the results in this study demonstrate that sorbic acid can have a significant inhibitory effect on the growth of *S. cerevisiae* without any appreciable reduction in pH_i (Figures 3.11 and 3.12 a)). Thus concluding, if direct pH_i reduction is not responsible for the inhibitory action of sorbic acid in yeast then there must be an alternative explanation.

To gain further understanding into the potential role of pH_i in growth inhibition, a comparison was performed of the inhibitory kinetics obtained with sorbic acid to those induced by exposure to amphotericin B. Despite inhibiting growth with similar kinetics to sorbic acid, the inhibitory activity of amphotericin B did correlate with reduction in pH_i at the lower pH values (Figures 3.10 b) and 3.11). Amphotericin inhibition was also highly dependent on the external culture pH (Figures 3.10 b),

3.11 and 3.12)), as all these results show the lower the external pH, the greater the inhibition of growth and the greater the decline in pH_i. Furthermore, exposure to a constant concentration of amphotericin B at decreasing pH values resulted in alteration of membrane permeability leading to increasing proton influx into the cell as the external pH dropped (Figure 3.13).

Therefore, the observed inhibitory effect of amphotericin B is consistent with gross membrane disruption (as shown by Palacios & Serrano, 1978), or proton-uncoupling, leading to growth inhibition due to proton influx and decline in pH_i. Hence, the observations of greater inhibition of growth at lower culture pH values. Exposure to a constant concentration of undissociated sorbic acid had the same effect on membrane permeability as amphotericin B except little change in pH_i was observed (Figure 3.13).

Although large reductions in pH_i in cells resuspended in growth media were not detected on sorbic acid addition, other studies have reported sorbate induced decline in pH_i in yeast (Cole & Keenan, 1987) and *Escherichia coli* (Salmond *et al.,* 1984) at similar pH values. However, in these published studies, pH_i was measured in cells that were in a non-physiological state, resuspended into buffers. Indeed, experiments in this study have shown that exposure of non-growing cells in buffer to sorbic acid did result in a detectable reduction in pH_i (Figure 3.6 and 3.7). Nevertheless, using the same methodology with growing cells under physiological conditions, the results do not show the same large sorbic acid induced reduction in pH_i.
A possible hypothesis to explain the above observations could be that sorbic acid would result in a detectable disruption in pH_i in the absence of any other homeostatic mechanism, e.g. induction of an energy-dependent stress response that could counteracts this detrimental effect.

There are two major pieces of evidence that support this hypothesis. Firstly, there is considerable evidence that sorbic acid does affect pH_i homeostasis due to the frequent observations that exposure to sorbic acid, and others organic acids, results in significant and rapid activation of the H^+-ATPase (Holyoak *et al.,* 1996, Piper *et al.,* 1997, Carmelo *et al.*, 1997 and Viegas & Sa-Correia, 1991) which is used by growing yeast cells to regulate and maintain pH_i homeostasis. Secondly, the results in

Figure 3.13 show that a membrane permeabilising effect is demonstrated on exposure to sorbic acid, which is similar to that induced by amphotericin B. This exposure thus resulted in an increasing proton influx across the membrane as the external pH declined and this effect we would expect to reduce the pH_i.

The available evidence supports this thesis's findings that the energy-state of the cells provides the underlying mechanism responding to protect the cells from the inhibitory effect of sorbic acid. The fact that disruption of pH_i homeostasis was not detected in this study does not necessarily mean that this was not taking place. The increased activation of the membrane H^+-ATPase, that is known to occur upon exposure to sorbic acid in growing cells (Holyoak *et al.,* 1996 and Piper *et al.,* 1997), may have resulted in the restoration of pH_i homeostasis such that any fluctuation became less detectable.

If this hypothesis is true, then how can the inhibitory effect of sorbic acid be explained?
Research has shown that coupling ATP hydrolysis to proton translocation can result in the membrane H^+-ATPase consuming up to 60% of total cellular ATP (Serrano, 1991). Thus importantly, this increased activity of the membrane H^+-ATPase is energetically expensive and such a depletion of ATP may restrict growth. Studies by Viegas & Sa-Correia (1991) have shown that activation of the membrane H^+-ATPase by octanoic acid correlated with a decrease in the biomass yield and Holyoak *et al.* (1996) have shown that a glycolytic mutant, with approximately one-third the normal pyruvate kinase and phosphofructokinase activity and hence a reduced ability to generate ATP, was more sensitive to sorbic acid.

Further results from this study have shown that on exposure to sorbic acid the intracellular ADP / ATP ratio increased (Figure 3.14). These results are in agreement with previous observations that also showed large reductions in the levels of intracellular ATP during growth in the presence of sorbic acid (Holyoak *et al.*, 1996). Thus, the inhibitory activity of sorbic acid can be explained as the induction of an energy-consuming response to the disruption of pH_i homeostasis that reduces cellular energy pools to the extent that there is less free energy for growth and division.

Evidence also suggests that this energy-demanding stress could also be due to the induction of plasma membrane pumps for active extrusion of weak acids from the cell enabling adaptation to such environment (first proposed by Warth, 1977 supported by data of Henriques *et al.*, 1997). Indeed, a pump recently identified as the Pdr12 protein is induced by sorbic acid in *S. cerevisiae* (Piper *et al.*, 1998) and study of this deletion mutant of Pdr12 has shown that active extrusion is important for resistance to sorbic acid (Piper *et al.*, 1998).

To discuss further the membrane permeability observations seen for sorbic acid (Figure 3.13), the studies of Weber & de Bont (1996) have shown that actives that are highly lipophilic such as amphotericin and to a lesser extent sorbic acid, partition in the membrane causing an increase in membrane permeability. This in turn increases the flow of protons and other ions across the membrane, leading to the dissipation of the proton motive force and resulting in less effective energy transduction. Other effects as reduction in activity of key membrane bound enzymes and loss of important metabolites can together cause major impact on cellular activity.

Thus whilst the results for amphotericin B indicate that the complete uncoupling of the membrane by the formation of stable membrane pores (Bolard, 1986) and thus means that any increased activity of the H^+-ATPase attempting to restore pH_i homeostasis would be futile. In contrast, although this study has shown that sorbic acid does induce membrane disruption this does not uncouple the membrane sufficiently to prevent increased H^+-ATPase activity maintaining pH_i homeostasis.

In summary, the results from Chapter 3 and other supporting evidence suggest that the growth inhibitory effect of sorbic acid on *S. cerevisiae* could be due the disruption of a number of different cellular activities rather than one. The inhibition seen may arise from not only the effect of free proton and anions liberated upon the dissociation of the acid in the cell, but also from the membrane disruption and inhibition of key cellular enzymes and processes as a result of this disruption discussed above. All of these systems resulting in different energy-consuming stress responses, attempting to restore pH_i and cellular homeostasis.

6.4. Proteomic analysis of yeast cytosolic proteins in the BY background identified five up-regulated proteins in response to sorbic acid

It was clear from the studies in Chapter 3 that there were numerous different cellular systems inhibited by the action of sorbic acid, and a number of different molecular models were being formed (such as that described by Piper *et al.*, 2001 and Figure 1.2). Thus to study these systems further this thesis used the approach of the proteomic 2D-PAGE, using both traditional staining and pulse labelling with ^{35}S methionine to identify cytosolic proteins with altered expression due to the exposure to sorbic acid (Chapter 4). It was hoped that by identifying new proteins involved in the stress response would provide new key factors that could be involved within the proposed model for weak acid resistance in yeast (Figure 1.2). By studying samples taken after a short time frame after sorbate addition, the proteins identified would be those that had undergone an immediate change in expression.

The results of the proteomic study showed that of those proteins excised and analysed by peptide mass fingerprinting, about half were shown to be of interest for further study. The combination of the Mowse score, experimental verses reported molecular weight and pI co-ordinates and using information regarding the proteins possible function within the stress response enabled a further short listing of proteins. In addition to this information, a post comparative analysis was preformed comparing published experimental data using other genomic and proteomic techniques (Schuller *et al.*, 2004, Mollapour *et al.*, 2004 and de Nobel *et al.*, 2001) and analysing their possible functions from the literature. This allowed a further short listing of candidate proteins with a higher degree of certainty of their involvement in the weak acid stress response. Other studies such as de Nobel *et al.* (2001), also used a similar proteomic approach to study the effect of sorbic acid on yeast, but the cells were harvested after reaching late exponential phase and the de Nobel study differed to this thesis methodology in that the identified proteins were involved in the adaptation to the stress rather than an immediate stress response.

The clarity and quality of the separation gave further evidence and confidence in the matches found and it was concluded to be of a good standard when compared to other published studies (Boucherie *et al.*, 1995, Blomberg, 1997 and Rabilloud *et al.*,

2001). Furthermore, the numerous repeats conducted over a number of data sets ensured that if any of the proteins of interest were of low abundance (discussed in depth by Navarre *et al.*, 1995), a correlation across the whole set gave confidence that the find was real and not an artefact.

It is clear from the diversity of proteins within the list that as a result of a stress from sorbate there is no one single cellular response, but a great complexity of different systems protecting the cell. This result agrees with the previous findings and conclusions from this thesis, that there are numerous cellular systems affected by weak acid addition. This list consisted of 10 proteins that were of potential interest for phenotypic deletion mutant analysis.

The proteins that were chosen for further phenotypic analysis were Ach1p (Acetyl-CoA hydrolase - spot 48), Cys4p (Cystathione beta-synthase - spot 68), the hypothetical protein Hcr1p (YLR192C - spot 187) and Cph1p (cycophilin peptidyl propyl cis-trans isomerase – spot 315). In addition the protein Hsp26p (heat shock protein 26 - spot 86) which was less well matched but had overwhelming evidence of its involvement in the stress response was also chosen. The possible eleven candidate cytosolic proteins identified in Chapter 4 that were up-regulated in response to sorbate stress and of this group, only five were chosen for further phenotypic analysis. These either having high correlation to other studies conducted or having a highly relevant role in stress resistance. Proteins that were identified as general maintenance proteins, for example, those that are involved with glycolysis and other general biochemical pathways, were not chosen for further study due to already extensive studies completed upon them (Busa & Nuccitelli, 1984 and de Nobel *et al.*, 2001). Even though some of these general maintenance proteins were shown to have involvement in the stress response, and indeed published results demonstrate this to the case and fits with the energy requirement hypotheses surrounding the stress response, these were not chosen for the first round of analysis.

6.5. The identified up-regulated proteins were not sorbate sensitive when analysed using phenotypic deletion mutant analysis

The five chosen proteins that were found to have a changed level if expression in response to the weak acid stress were investigated further via phenotypic deletion mutant analysis to identify any that could be possible proteins involved in stress resistance.

The gene deletion mutants were obtained for the proteins in question but were only available in a BY genetic background (haploid parental strain BY4741) from the *Saccharomyces* Genome Deletion collection. This parental background being almost genetically identical to that of the FY parental strain used in the proteomic (Chapter 4) and pH_i studies (Chapter 3), although the only difference is that the BY strain is TRP^+ and the FY strain *trp-*.

Contrary to expectation, the phenotypic evaluation of these gene deletion mutants revealed that none were sensitive to sorbic acid. Two of the deletion strains, *hcr1* (a gene without known function) and *cys4* (a mutant defective in biosynthesis of cysteine) showed very slight sensitivities to the weak acid, however, these results were not consistent across the tests performed (*hcr1* showed an increased level of sensitivity in one test - Figure 5.2, and the *cys4* mutant was slightly more acid sensitive in another test - Figure 5.3). It can be therefore concluded that these findings hold inconsistencies and indeed no mutant strain demonstrated and overwhelming weak acid sensitivity.

These results were surprising especially for the deletion mutants *ach1*, *cys4*, *hsp26* and *cph1* in particular, all of which had correlated well to other studies. Furthermore, the acid sensitivity results did not agree with other findings (Mollapour *et al.*, 2004, de Nobel *et al.*, 2001 and Schuller *et al.*, 2004) that did show sorbate sensitivity for these deletion mutants. Thus these inconsistencies would need further investigation, although would not be performed in this thesis due to the direction of the research taken.

6.6. Observed differences in sorbate sensitivity between the FY and BY parents

The above findings showed that there were clearly major inconsistencies with the findings between the proteomic study and of the phenotypic analysis. One observation that was discovered was that the two genetically almost identical strains FY1629-28c and BY4741 when compared had two very different sorbate sensitivities. The FY strain was considerably more sorbate sensitive than the BY strain, the addition of sorbate significantly inhibiting the growth of the FY strain compared to that of BY (Figure 5.4). The only major difference between these strains is their genetic backgrounds, FY1629-28c is a tryptophan auxotroph (*trp-*) and BY4741 a tryptophan phototroph (TRP^{+}). Thus this difference was investigated further to whether auxotrophy for tryptophan could be enhancing this observed weak acid sensitivity.

This result raised questions with regard to the use of an already sorbate sensitive FY parent in the proteomics study in Chapter 4, could have possibly given different and heightened expression responses that may not have been seen in the BY parent and thus giving conflicting data in the phenotypic analysis.

6.7. Characterisation of sorbate sensitivity within tryptophan auxotrophic mutants

To investigate the effects of defects in tryptophan biosynthesis on weak acid resistance, five deletion mutants for enzymes of the tryptophan biosynthesis pathway in the BY4741 background were investigated. These mutants when studied in the presence of both sorbate and acetate weak acid stress, resulted in all of the Δ*trp* mutants being more sensitive to the weak acids than the wild type BY parent (Figure 5.7). All of these mutants, unlike the BY parent, need to catalyse the uptake of tryptophan from the medium in order to grow, as one steps in their tryptophan biosynthesis pathway is blocked.

Suspecting that the weak acids were strongly inhibiting the uptake of tryptophan, the effects of supplementing the medium with high levels of tryptophan was

investigated. Whilst the *trp5Δ* mutant was extremely sorbate sensitive, this hypersensitivity was almost totally suppressed by the high levels of tryptophan (Figure 5.6). Such supplementation restored the growth of the weak acid stressed *trp5Δ* mutant cells to that of the control, stressed wild type BY (*TRP*$^+$) cells lacking such supplementation.

Similar results were obtained when using the same strains with acetate, and again, this hypersensitivity was substantially suppressed with the addition of high levels of tryptophan supplementation (Figure 5.9). Thus concluding that indeed the tryptophan biosynthetic mutants are weak acid sensitive, but this sensitivity can be restored by adding extra tryptophan.

These findings were also in agreement with the later findings of Mollapour *et al.* (2004) and Schuller *et al.* (2004) who both identified the tryptophan biosynthesis deletion mutants as being sorbate sensitive (both conducting their studies in the BY (*TRP*$^+$) background). However, the studies of de Nobel *et al.* (2001) did not identify any of these *trp-* mutants, the fact that they conducted the screen in the more sorbic acid sensitive FY *trp-* parent genetic background could provide an explanation in the differing results.

As a result of the above data, it was hypothesised that an increased capacity for the *trp-* cells to catalyse uptake of aromatic amino acids might suppress this weak acid sensitivity. To obtain further evidence for this hypothesis, Bauer *et al.* (2003) studied the effects of over-expressing the high affinity tryptophan permease, Tat2p (Schmidt *et al.*, 1994). The increased sorbate sensitivity due to *trp1-Δ63* mutation was substantially suppressed by the p*TAT2* over-expression vector, a plasmid which could almost restore the growth of the sorbate-stressed *trp1-Δ63* cells to the level of growth displayed by an isogenic *TRP*$^+$ prototroph (Fig. 5.8 a)). Thus providing further evidence that the tryptophan auxotrophy was causing this extreme weak acid sensitivity.

6.8. Determination of differing cellular responses to weak acids of the same deletion mutants be due to tryptophan auxotrophy

A potential weak acid resistance model discussed in this thesis (Figure 1.2) and by Piper *et al.* (1998), discuss the involvement of a plasma protein; a transporter protein Pdr12 to be strongly induced after exposure of *S. cerevisiae* to sorbic acid (Piper *et al.,* 1998). The sorbate induction of Pdr12p is so strong, that this becomes one of the most abundant plasma membrane proteins (Piper *et al.,* 2001). Cells lacking the Pdr12 transporter (the *S. cerevisiae pdr12* mutant) are hypersensitive to water-soluble monocarboxylic acids of relatively short aliphatic carbon chain length (Piper *et al.,* 1998 and Holyoak *et al.,* 1999). They are also sensitive to short chain alkanols (n-propanol, n-butanol and n-pentanol) (Hatzixanthis *et al.*, 2003). It appears therefore that Pdr12p imparts resistance to those organic acids or alcohols that can, to a reasonable degree, partition into both lipid and aqueous phases (Holyoak *et al.,* 1999 and Holyoak *et al.,* 2000).

However, research by Piper *et al.* had discovered contradictory results when studying acetate sensitivity in *pdr12* and *azr1* mutants. Earlier work had shown that the mutant strain *pdr12*Δ was more acetate sensitive than the wild type (Piper *et al.*, 1998) and a separate study had reported that Azr1p, a plasma membrane transporter of the major facilitator superfamily, also conferred acetate resistance (Tenreiro *et al.*, 2000).

But later studies using a different strain background which contained no auxotrophic requirements for aromatic amino acids (i.e. were TRP^{+}) showed this time the loss of Pdr12p or Azr1p resulted in no increased sensitivity to acetate. Instead, the loss of Pdr12p alone in this background resulted in slightly increased resistance to acetate (Bauer *et al.*, 2003).

This thesis sort to determine whether or not Pdr12p was indeed induced as a result of the presence of weak acid and thus confirm its role in the suggested weak acid model by Piper *et al.*, 2001. Chapter 5 described the study to determine which of these proteins, Pdr12p or Azr1p, were more important for acetate resistance (Bauer *et al.*, 2003) due to the observation that losses of Pdr12p or of Azr1p appeared to be

exerting opposite effects on acetate resistance in *trp-* and *TRP*$^+$ genetic backgrounds. As from previous results clearly the auxotrophic background causing profound differences in weak acid sensitivity.

Studies were conducted to investigate the effects of the loss of Pdr12p and Azr1p on acetate resistance in *TRP*$^+$ cells. The results in Figure 5.9 show that the losses of these genes result in either neutral effects or a decreased sensitivity to acetate in *TRP*$^+$ cells, whereas in *trp-* mutants the losses of these transporters increased sensitivity to acetate.

To study these results further Bauer *et al.* (2003) investigated the effects of high levels of Pdr12p induction on acetate resistance by using the strain *GAL1-PDR12,* where the *PDR12* gene was placed under the control of the *GAL1* promoter because the *PDR12* gene is not normally induced by acetate (Hatzixanthis *et al.*, 2003). However, during growth of this *GAL1-PDR12* strain on galactose media, *PDR12* can be induced to levels similar as to those seen in wild type cells exposed to sorbic acid stress (Hatzixanthis *et al.*, 2003). Results in Figure 5.10 showed that growth of normal wild type yeast on galactose relative to glucose slightly decreased acetate resistance, yet the strong Pdr12p induction with the growth of the *GAL1-PDR12* strain on galactose decreases acetate resistance still further. This result contrasts greatly with that for sorbate, where an increased resistance results from the same *GAL1* promoter-directed induction of Pdr12p (Hatzixanthis *et al.*, 2003). This provided further evidence that the induction of Pdr12p, though beneficial for resistance to C_{3-8} aliphatic carboxylic acids and to sorbate (Piper *et al.,* 1998 and Hatzixanthis *et al.*, 2003), is actually somewhat detrimental for resistance to acetate.

Unpublished work by M. Mollapour has shown that neither Pdr12p nor Azr1p is actually induced by acetate stress although a *GAL1* promoter-directed induction of Pdr12p clearly results in a reduced resistance to acetate (Figure 5.10).

These results appeared to contradict the earlier findings by the group that loss of Pdr12p resulted in a decrease in acetate resistance (Piper *et al.*, 1998). However, the strains used in these earlier studies were identical to those used to obtain the data in Figure 5.9 and 5.10, except that they possessed the additional *trp1-Δ63* mutation. When the *trp*1-*Δ63* strains used in earlier work were re-tested (Piper *et al.*, 1998),

they were found to be generally less weak acid resistant than the corresponding *TRP*$^+$ strains and thus explaining these observed differences.

Further control experiments (Bauer *et al.*, 2003) showed that the high tryptophan levels had no effect on Pdr12p induction by sorbate (results not shown). Furthermore, because exogenous tryptophan suppresses both the sorbate (Figure 5.6 and acetate (Figure 5.7) and sorbate (Figure 5.8) sensitivities of *trp-* biosynthetic mutant cells (only the sorbate is a Pdr12p inducer) it is clear that this sensitivity suppression by exogenous tryptophan bears no relationship to the weak acid inducibility of Pdr12p. Furthermore, Pdr12p levels were not affected by *TAT2* over-expression, indicating that this rescue of acid-stressed cells by pTAT2 acts independently of the Pdr12p transporter. Tat2p over-expression could partially rescue the sorbate sensitivity of the *war1-42* mutant, a mutant lacking Pdr12p induction, in a *trp1* genetic background (Figure 5.8 b)).

Bioscreen analysis of *Δpdr12* and *Δazr1* single gene deletes in the *TRP*$^+$ backgrounds (Figure 5.11) confirmed that loss of Azr1p alone did not increase the sorbate sensitivity of *TRP*$^+$ cells, though this loss increased the sorbate sensitivity of the *Δpdr12* mutant (Figure 5.11 b)). Unexpectedly, the *Δpdr12* and *Δazr1* single gene deletes grew considerably better than the wild type at pH 4.5 in the presence of 120 mM acetate (Figure 5.11 d)). After an extended lag phase, the *Δpdr12 Δazr1* double mutant also grew considerably better than the wild type. Loss of either of Pdr12p or Azr1p appears therefore to be beneficial for growth in the presence of high concentrations of acetate.

The reasons for this improved growth are not clear, but one possibility is that these mutants are not displaying the apoptotic events normally seen in yeast cells treated with high levels of acetate (Ludovico *et al.*, 2001). It is therefore unlikely that Pdr12p or Azr1p confer acetate resistance as suggested by earlier work (Piper *et al.*, 1998) indeed it appears that their loss may actually elevate this resistance (Figure 5.11).

In summary, it has been shown that aromatic amino acid auxotrophic strains are hypersensitive to sorbic acid, but this sensitivity can be repressed by either

supplementing extra tryptophan to the medium or the over-expression of a tryptophan permease in such *trp-* cells. This hypersensitivity has clearly caused conflicting data when comparing deletion mutants in different genetic backgrounds, the *trp-* background giving results indicating possible weak acid sensitivity where there is not.

The proteins identified in Chapter 4 proteomic study were done so using an FY parent strain that was *trp-* and the induced proteins identified as a result of this stress, could possibly not be sensitive to sorbate in a TRP^+ background. However, the results from Chapter 4 were compared to studies (Mollapour *et al.*, 2004 and Schuller *et al.*, 2004) that conducted similar sorbic acid stress analysis in BY TRP^+ background, thus giving confidence in the findings. However, for the work of de Nobel *et al.* (2001) this was not the case and had used the same FY *trp-* strain as in Chapter 4. In particular there was only one protein that was only identified by de Nobel *et al.* (2001) and this was *cph1* (cycophilin peptidyl propyl cis-trans isomerase) and it is possible that for this deletion mutant in particular the *trp-* background gave it a hypersensitive phenotype that was not seen in the TRP^+ background.

The differences in the patterns of gene expression seen Mollapour *et al.* (2004) compared to the results of de Nobel *et al.* (2001) were commented upon. It was hypothesized that the differences could be due to the work of de Nobel was investigating adaptation over a longer period of time rather than that of resistance over a short period of time. However, Mollapour *et al.* (2004) alluded to a possible issue with conducting such studies in a *trp-* background could give arise to different expression patterns. In deed this study confirms the hypothesis that such a *trp-* background would give rise to a set of genes sensitive to weak acid that would not normally be so under TRP^+ conditions. These results thus explaining the lack of correspondence between the changes incurred as a result or weak acid stress. However, upon studying the results of Mollapour *et al.*, (2004) it is clear just the extent and complexity that the weak acid response has upon a cell and the systems that are induced to cope with the stress. As discussed earlier in these conclusions, it is not only intracellular pH that is changed but the homeostasis of numerous systems

are affected and the cell seeks to re-adjust and regain control over each of these in order to survive.

By studying some of these key systems we can seek to understand the overall complexity that weak acids have on the cell and the numerous systems the cells initiates to combat this stress.

With these results in mind, the findings of *pdr12* mutants were assessed to determine if the weak acid sensitivity seen for this mutant with sorbate and acetate was the case. This study confirmed that Pdr12p is still one of the integral elements in the response to resistance to sorbic acid, however, it is not required for acetate resistance which was concluded earlier in *trp-* background strains.

It is therefore expressed, that care is required when conducting any kind of stress assessment with deletion mutants. The choice of parent strain with regard to the choice of the genetic background is imperative so that it does not impose additional bias to the results.

Summary of conclusions from this thesis:

- ***A fluorescent in vivo intracellular pH method was successfully developed and the resulting data was comparable to published results***

- **A *threshold intracellular pH does not need to be achieved in growing yeast cells before outgrowth from the lag phase can occur***

- ***The addition of weak acid to exponentially growing yeast cells appears not to cause observable disruptions in pH_i even when growth rates and the membrane permeability's are clearly altered. However, sorbic acid would result in disruption in pH_i in the absence of any other homeostatic mechanisms, but these changes would be suppressed by the initiation of a number of different cellular responses***

- ***Proteomic analysis of yeast cytosolic proteins in the FY background identified five to be up-regulated in response to sorbic acid, but phenotypic analysis of the deletion mutant strains in a BY background showed that none were found to be sorbate hypersensitive. Further analysis showing that the FY genetic background (trp- phenotype) is far more weak acid sensitive than the BY genetic background (TRP^+ phenotype). Auxotrophic requirements for aromatic amino acids cause unusually high sensitivity to weak organic acid stress but this sensitivity can be reverted by the supplementation of tryptophan to the growth media***

- ***This study confirmed that Pdr12p is still one of the integral elements in the response to resistance to sorbic acid (showing sensitivity in both trp- and TRP^+ backgrounds). However, it is not required for acetate resistance which was concluded earlier in trp- background strains.***

- ***Care is required when conducting any kind of stress assessment with deletion mutants. The choice of parent strain with regard to the choice of the genetic background is imperative so that it does not impose additional bias to the results.***

Chapter 7 – Future Work

At different points in this thesis a number of different research directions could have been taken. For instance at the end of the fourth and beginning of the fifth chapter the findings indicated that there was clearly an issue with using different phenotypic backgrounds within a proteomic screen. As such at this point one direction could have been to perform the proteomic screen again in the BY (*TRP*$^+$) background looking for differences in response to sorbic acid, or alternatively to investigate the inconsistencies between the results in this thesis and published results for the mutants. However, it was decided to study further the specific reasons to why auxotrophy to aromatic amino acids causes the sensitivity seen for some weak acids and not others.

Thus the obvious future work direction would have been to go back and repeat the proteomic study in the BY background in the presence of sorbic acid. However, due to the time span of this thesis and the pace of research in this area, other colleagues from the group using the significant technological advances in the field embarked on a major study to investigate the impact of weak acids on the yeast genome and proteome in the BY (*TRP*$^+$) background (Mollapour *et al.*, 2004 and Schuller *et al.*, 2004). These studies have resulted in a huge amount of valuable information, some having a clear impact on the findings of this thesis and thus were retrospectively incorporated into the applicable discussions in Chapters 4 and 5.

As a result of this thesis and the work of Mollapour *et al.* (2004) and Schuller *et al.* (2004) a number of different areas of research were identified that could be involved in the resistance to weak acids in yeast. The further study into any number of these areas could possibly give increases understanding into possible additional systems that respond to give weak acid resistance in yeast. However, the results of Mollapour *et al.* (2004) have shown the sheer complexity of the different systems involved and the inhibition of these clearly all contribute to the cessation of growth. The following areas identified discussed in this Chapter have been chosen as being of interest to further the understanding into weak acid resistance in yeast.

7.1. Sorbic acid interactions with the yeast cell membrane

The studies within Chapter 3 show that sorbic acid like amphotercin can to some extent cause a level of membrane permeability. Indeed the very lipophilic nature of sorbic acid may be more inhibitory than other weak acids due to its increased ability to partition into the membrane structure and the accumulation of such compounds would undoubtedly affect the physico-chemical and consequently the function of the membrane, as well as liberating free anions and protons into the cell. The accumulation of organic solvents has been shown to affect the permeability of the membrane, but also disorder the membrane structure with possible deleterious effects on the conformation of key membrane bound enzymes - all having a negative effect on the cell (Weber & de Bont, 1996). Other weak acids have been shown to differ in their exhibited effect by purely acting as a typical weak acid, and being less hydrophobic are less able to partition into the membrane (Piper *et al.*, 2001).

Such hypothesis have been built upon for instance by Piper *et al.,* 2001, to explain the role of Pdr12p and its ability to give resistance to short chain alkanols, compounds whose toxic effects are thought to be due mainly to their ability to dissolve in membranes (Weber & de Bont, 1996). Piper *et al.,* 2001 suggests that this ABC transporter may bind acid anions or alcohol molecules actually incorporated in the inner leaflet of the plasma membrane. One can surmise that Pdr12p then transports these to the opposite (periplasmic) side of the membrane, in order to release them into the aqueous phase of the periplasm. Such active efflux may be able to lower the intracellular level of the acid anion or alcohol, on the basis that the polar groups on these carboxylate anion or alcohol substrates will slow their diffusion back across the cell membrane.

Thus the combined actions of the H^+-ATPase and Pdr12p may both be needed to restore homeostasis to the point where the yeasts cells can grow. Both processes are undoubtedly expensive in energy terms and thus would be reflected in dramatic reductions in biomass yield. Such anion and proton expulsion would seem pointless without some system restricting free diffusional entry of the undissociated acid (XCOOH). However, how weak acid diffusion is restricted in adapted cells at present remains unknown (Piper *et al.,* 2001).

Possible thoughts and ideas as how the yeast cell could restrict this diffusion can be found in the work of Weber & de Bont (1996). A number of adaptive changes which result in the membrane composition of yeast as a result of accumulation of organic solvents are discussed. These changes in the membrane composition could be induced to counteract the effects induced by these solvent and by maintaining the optimal order of the lipids in the membrane can stabilise as well as reduce solute partitioning. Weber & de Bont (1996) discuss that a number of their studies have shown that solvents increase the permeability of the membrane by changing the lipid fluidity.

Such changes as changing the sterol content of a membrane can drastically affect the fluidity. A decrease in ergosterol content of the yeast cell membrane has been directly related to a decrease in cell viability in the presence of ethanol. Similarly, supplements of ergosterol enhance the cell viability in the presence of ethanol.
To add further evidence to this hypothesis, the involvement of ergosterol in the cell membrane adaptation to stress environments is provided by the work of Mollapour *et al.* (2004). Where a number of genes were identified that were involved in the biosynthesis of ergosterol and when deleted from the yeast genome resulted in heightened weak acid sensitivity in yeast. Concluding that this altered sterol composition may facilitate the passive diffusion of the weak acid across the membrane.

Thus these results along with the work of Weber & de Bont (1996) add weight to the hypothesis and thus the need to study further this direction that cell in order to survive in such hostile environments as well as inducing the H^+-ATPase and PDR12p to extrude anions an protons the cell must also change its membrane to restrict entry.

7.2. The involvement of the protein tryptophan permease (Tat2p) with weak acid stress

As a result of the findings in this thesis and the effect seen in the weak acid response and ability to synthesise aromatic acids under such stress, other studies have been compared. Benov & Fridovich (1999) have shown there to be differences between the prokaryotic and eukaryotic responses to synthesise aromatic amino acids during weak acid stress. For instance *Escherichia coli* cannot synthesis these aromatic amino acids when suffering from severe oxidative stress. This auxotrophy in the cell seems to be the result of oxidation of the 1,2-dihydroxyethyl thiamine pyrophosphate intermediate of transketolase, which is suppressed when cultures are supplemented with intermediates (e.g. shikimate) allowing aromatic amino acid synthesis to occur independently of the transketolase reaction.

However, Bauer *et al.* (2003) concludes, that weak acid stress in yeast is acting in a fundamentally different way. It is not generating an auxotrophy for aromatic amino acids in wild type cells, but rather is causing high sensitivity to any requirement for the cells to catalyse uptake of aromatic amino acids from the medium. This could be due to the weak organic acids exerting a strong inhibition of the activity of the tryptophan amino acid permease in yeast (Tat2p), though this is not directly proven by this thesis.

This strong inhibition could be due to the severe energy depletion within cell caused by the weak acids disrupting pH_i balance and inhibit the possible energy dependant active transport activity of this specific high affinity permease, Tatp but also perhaps affecting the general amino acid permease, Gap1p. Both of these enzymes may need ATP to catalyse the uptake of aromatic amino acids, but with the cell using all of its available ATP to maintain homeostasis this could inhibit the transporters from any activity.

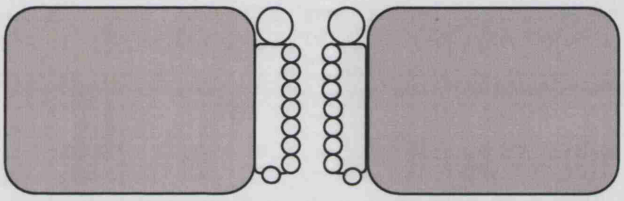
Although the studies of Mollapour *et al.*, 2004 did not identify Tat2p loss to cause any detectable sorbate sensitivity, it is clear that further study is needed to clarify Tat2p role in the weak acid sensitivity in a tryptophan auxotrophy.

Appendices

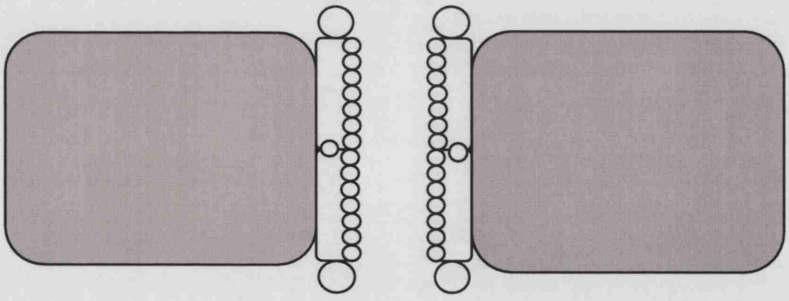
Appendices 1.0 – Theoretical model for Amphotericin B

The diagrams below depict a suggested model of the 'barrel' pore formation for amphotericin B within the cell membrane of yeasts and moulds (Bolard, 1986).
Figure a) depicts this cylindrical structure forming a half pore across the cell membrane in which an aqueous channel of approximately 8Å diameter is formed. Two such half pores when located opposite each other in the membrane form a conducting pore allowing free passage of ions and small molecules across the membrane (Figure b) (Hoogevest & Kruijff, 1978).

a) Half Pore

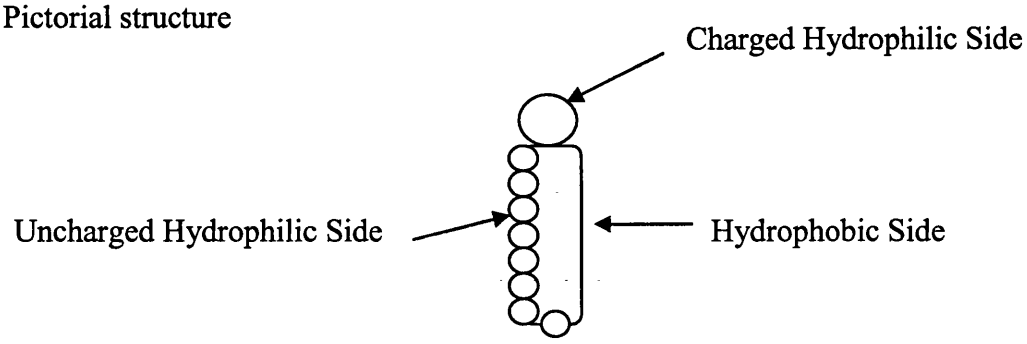


b) Double Half pore



The shaded part represents the hydrophobic cell membrane thus the hydrophobic charged side of amphotericin B will interact with.

c) Pictorial structure



The uncharged hydrophilic side of the molecule forms the internal part of the pore and thus interfacing with the aqueous environment. The charged hydrophilic side orientates itself towards either the inner or outer part of the cell also into the aqueous environment.

Taken from Hoogevest & Kruijff (1978).

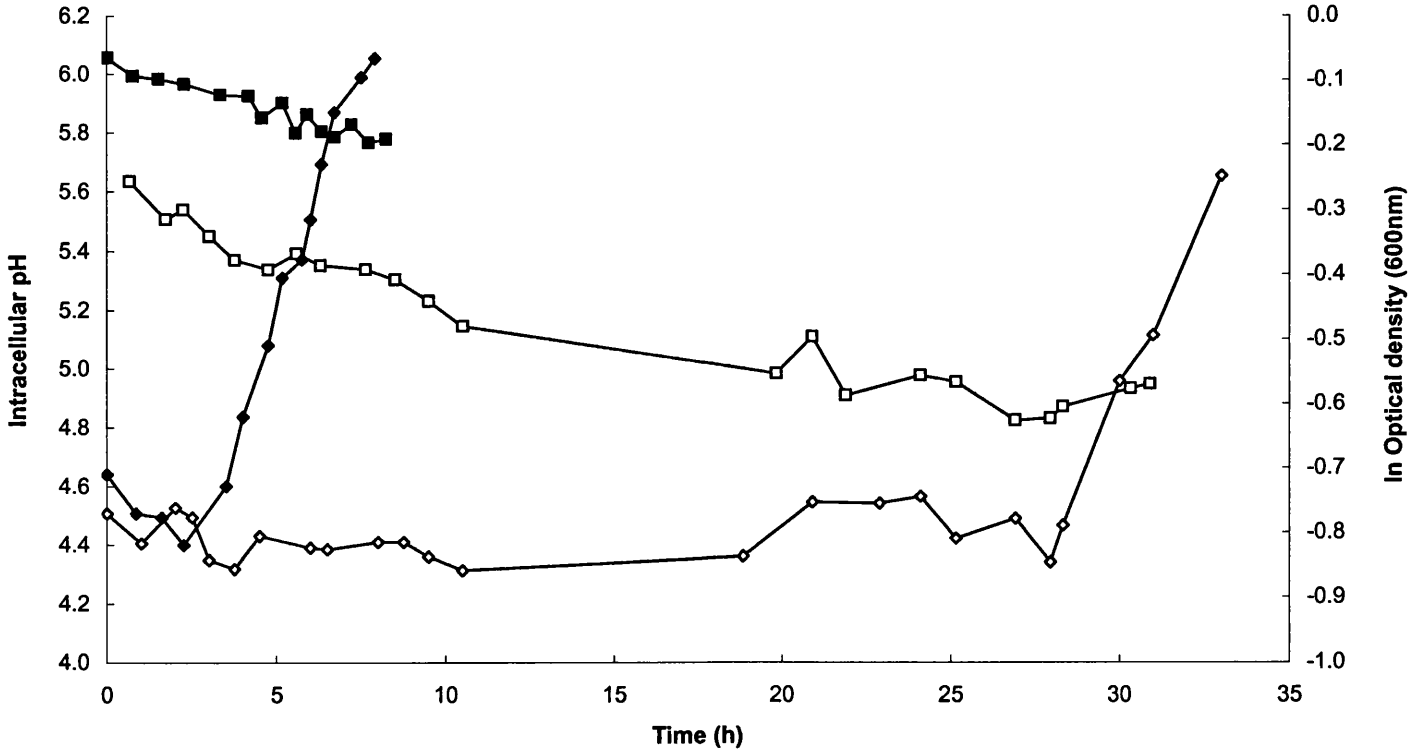
Appendices 2.0 – Supporting data and further examples of experimental results

Appendix 2.1 – Further example data supporting Figure 3.8

Supporting the data in Figure 3.8, another experiment was performed where pH_i and $O.D._{600}$ were simultaneously measured for growing cells of *PMA1* and *pma1-205* in YNBG-CP, pH 4.5 at 30°C, this time without the addition of any sorbic acid.

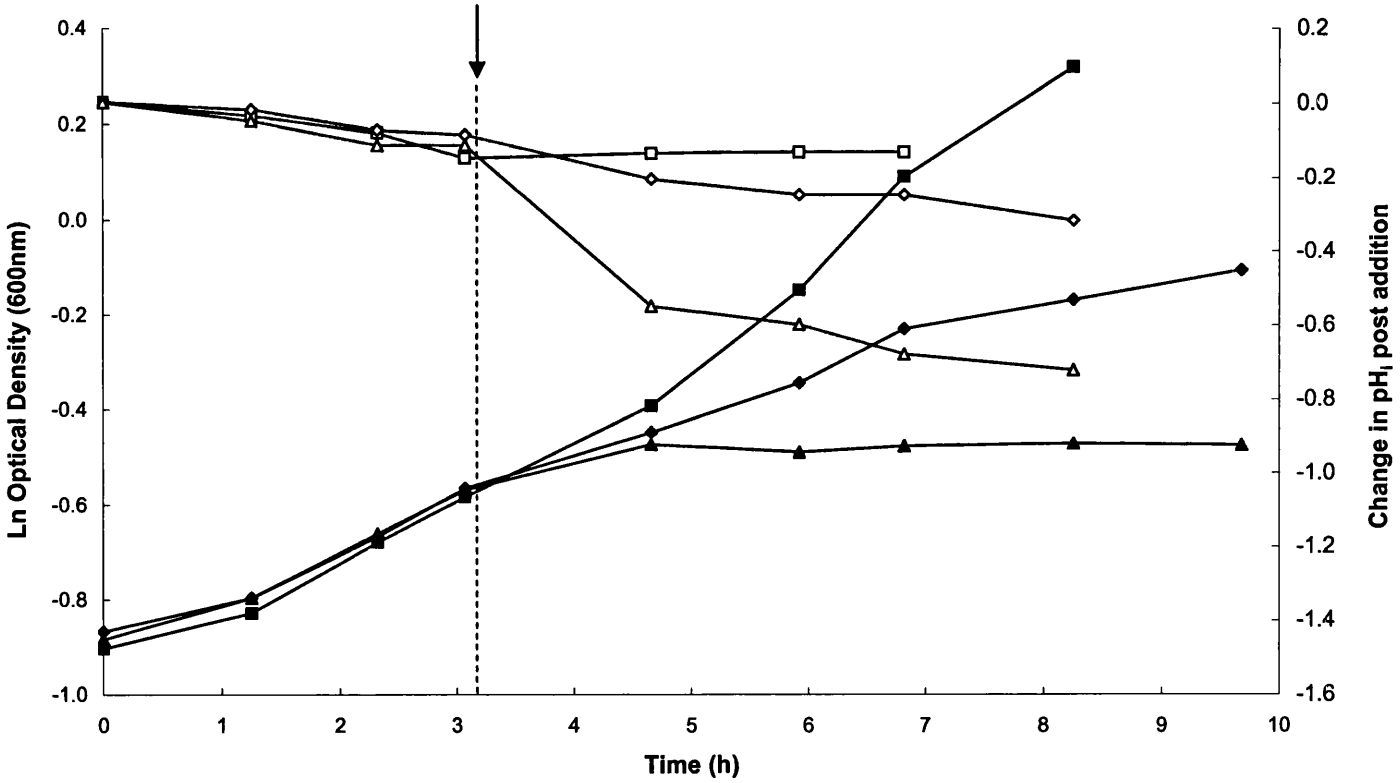
Appendix 2.2 to 2.4 – Additional experimental data supporting Figure 3.9

Figure 3.9 depicts increasing concentrations amphotericin B added to growing cultures of *S. cerevisiae PMA1* in YNBG-CP medium at pH 3.8. Similar experiments were carried out with sorbic acid at pH 3.8 and with sorbic acid and amphotericin B at a culture pH 4.5 and these results are shown in Appendices 2.2, 2.3 and 2.4.



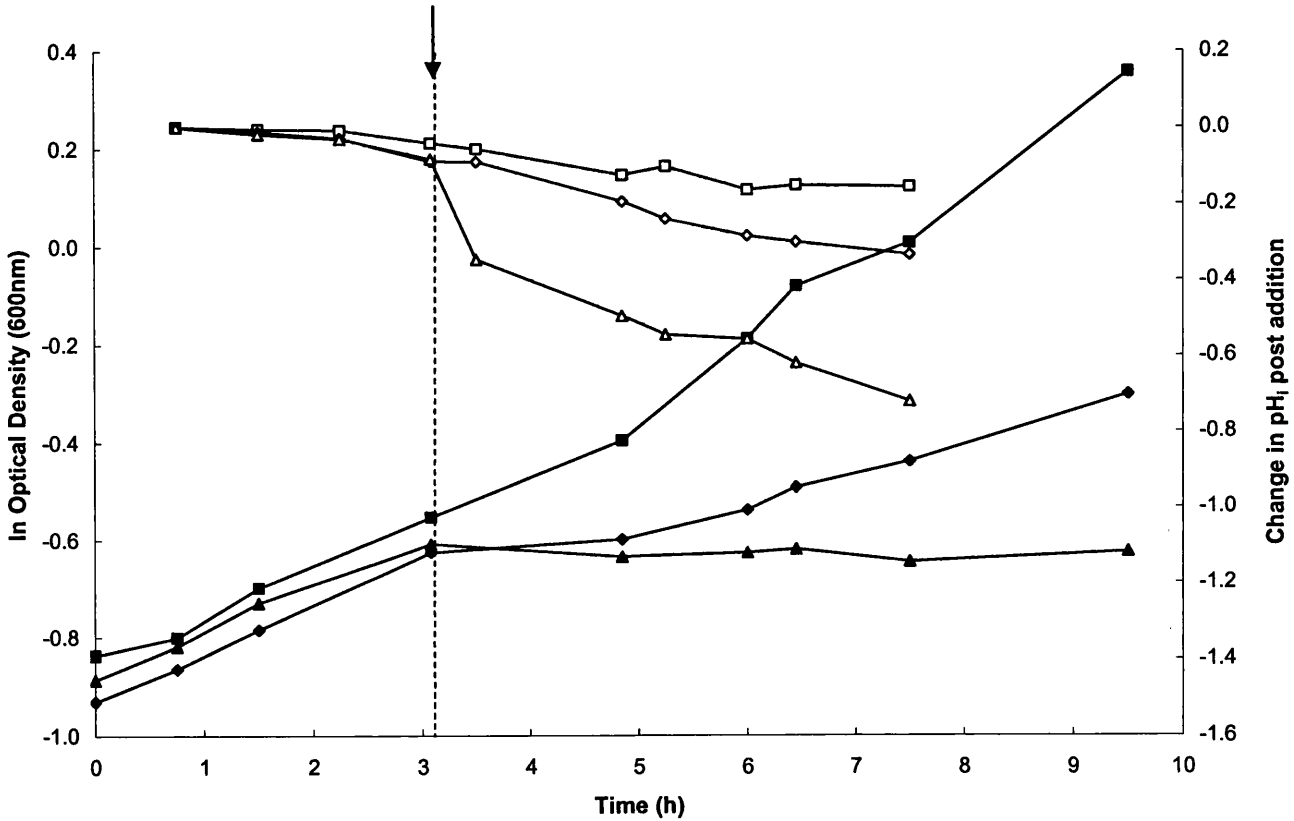
Appendix 2.1

Simultaneous measurement of logarithmic growth (ln optical density 600nm) (♦, ◊) and intracellular pH (■, □) of *S. cerevisiae PMA1* (solid symbols) and *pma1-205* (open symbols) in YNBG-CP, pH 4.5 at 30°C. This graph is representative of a typical plot.



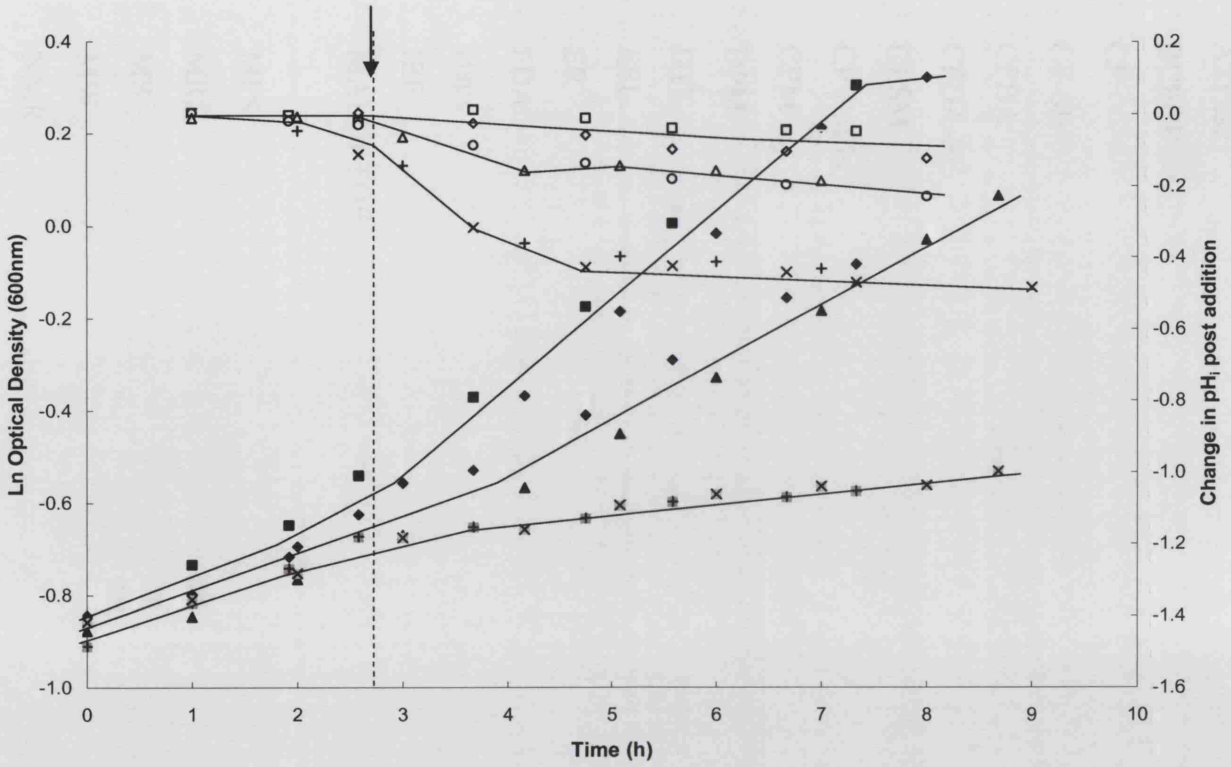
Appendix 2.2

The effect of addition of 0 (■,), 0.437 µM (♦, ◊) and 4.5 µM (▲, Δ) amphotericin B on the growth (solid symbols) and change in pH_i post addition (open symbols) of *S. cerevisiae PMA1* in YNBG-CP at pH 4.5. The dotted line and arrow indicates the point of addition of amphotericin B. The initial starting pH_i was approximately 5.8. The graph represents a typical plot.



Appendix 2.3

The effect of addition of 0 (■,), 0.25 mM (♦, ◊) and 0.75 mM (▲, Δ) sorbic acid on the growth (solid symbols) and change in pH_i post addition (open symbols) of *S. cerevisiae PMA1* in YNBG-CP at pH 3.8. The dotted line and arrow indicates the point of addition of sorbic acid. The initial starting pH_i was approximately 5.8. The graph represents a typical plot.



Appendix 2.4

The effect of addition of 0 (■,♦, , ◊), 0.25 mM (▲,●, Δ, ○) and 0.75 mM (X, +, X, +) sorbic acid on the growth (solid / shaded symbols) and change in pH_i post addition (open / unshaded symbols) of *S. cerevisiae PMA1* in YNBG-CP at pH 4.5. The dotted line and arrow indicates the point of addition of sorbic acid. The initial starting pH_i was approximately 5.85. The graph represents two experiments performed at different times over laid as scatter plots. The lines represent the average position for the two data sets.

Abbreviations

ABC	ATP-binding Cassette
ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
ATPase	ATP synthase
BCECF	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
CF	Carboxy Fluorescein
CF-SE	Carboxy Fluorescein Succinimidyl Ester
CFDA	Carboxy Fluorescein Diacetate
CFDA-SE	5-(6-) Carboxy Fluorescein Diacetate Succinimidyl Ester
CLSM	Confocal Laser Scanning Microscopy
CP Buffer	Citric / Phosphate Buffer
CPM	Counts per Minute
DPM	Disintegrations Per Minute
DTT	Dithiothreitol
ESI	Electro Spray Ionisation
ER	Endoplasmic Reticulum
FDA	Fluorescein diacetate
Hsps	Heat Shock Proteins
IEF	Isoeletric Focussing
MALDI-TOF	Matrix-assisted laser desorption / ionisation with time of flight tube
MFS	Major Facilitator Superfamily
MRD	Maximum Recovery Diluent
MS	Mass Spectrometry
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance
ORF	Open Reading Frame
PDR	Pleiotropic Drug Resistance
pH_i	Intracellular pH

pI	Isoelectric Point
pKa	Dissociation Constant
PMSF	Phenylmethanesulfonyl fluoride
SC	Synthetic Complete Media
SDS	Lauryl sulphate sodium salt (or Sodium dodecyl sulfate)
SDS-PAGE	SDS – Polyacrylamide Gel Electrophoresis
STRE's	Stress Response Elements
TCA	Trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine
YPD	Yeast Peptone Dextrose
YNB	Yeast Nitrogen Base
YNBG	Yeast Nitrogen Base Glucose
YNBG-CP	Yeast Nitrogen Base Glucose – Citrate Phosphate
2D-PAGE	Two Dimensional-Polyacrylamide Gel Electrophoresis

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Web-sites

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www.stanford.edu/group/yeast_deletion_project/deletion.html

SGD Yeast Database:
http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html

MIPS Yeast Genome Database:
www.mips.gsf.de/genre/proj/yeast

YPD Database:
www.proteome.com

Eurofan Deletion Project Website:
http://mips.gsf.de/proj/eurofan/

Euroscarf Collection Website:
http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html

Nationmaster Encyclopaedia Website
http://www.nationmaster.com/encyclopedia/Acetic-acid